THE JOURNAL OF EXPERIMENTAL MEDICINE

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

VOLUME THIRTY-THIRD

WITH ONE HUNDRED AND EIGHT PLATES AND
ONE HUNDRED AND SEVEN FIGURES
IN THE TEXT

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1921

COPYRIGHT, 1921, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S A.

JAN 7 192

THE JOURNAL OF EXPERIMENTAL MEDICINE

JAN 5 1921

EDITED BY

SIMON FLEXNER, M.D.

VOLUME XXXIII, NO. 1 JANUARY 1, 1921

PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1921, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Acceptance for mailing at special rate of postage provided for in Section 1103, act of October 3, 1917.
Authorized June 29, 1918.

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of between 500 and 600 pages. The subscription price is $3.00 per volume to subscribers in the United States and Canada, payable in advance; $3.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y. W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. It is issued bimonthly, one volume of about 600 pages appearing in a year. The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

CONTENTS.

No. 1, JANUARY 1, 1921.

PAGE

COWDRY, EDMUND V. The reticular material of developing blood cells. Plate 1 1

JONES, F. S. Streptococci occurring in sour milk 13

GATES, FREDERICK L. Preparation of collodion sacs for use in bacteriology 25

GATES, FREDERICK L. A mechanical measuring instrument for sterile liquids 45

GATES, FREDERICK L., and OLITSKY, PETER K. Factors influencing anaerobiosis, with special reference to the use of fresh tissue. Plate 2 51

SWIFT, HOMER F. Preservation of stock cultures of bacteria by freezing and drying 69

DRINKER, CECIL K., and SHAW, LOUIS A. Quantitative distribution of particulate material (manganese dioxide) administered intravenously to the cat. Plates 3 and 4 77

WADSWORTH, AUGUSTUS B., GILBERT, RUTH, and HUTTON, ALICE. Study of the classification of meningococci 99

WADSWORTH, AUGUSTUS B. Comparison of the potency of polyvalent antimeningococcus serum produced with four and six representative strains and that produced with sixty strains, as determined by the agglutination titer 107

WADSWORTH, AUGUSTUS B., and MALTANER, FRANK. Purification and concentration of antigens for complement fixation by methods of dialysis, adsorption, and extraction 119

No. 2, FEBRUARY 1, 1921.

OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. I. Transmission experiments with nasopharyngeal washings. Plates 5 to 7 125

392536

BOECK, WILLIAM C. *Chilomastix mesnili* and a method for its culture..... 147
OLIVER, JEAN. Mechanism of urea excretion. Plates 8 and 9. 177
AMOSS, HAROLD L. Immunological distinctions of encephalitis and poliomyelitis..... 187
PEARCE, LOUISE, and BROWN, WADE H. Therapeutic action of *N*-phenylglycineamide-*p*-arsonic acid (tryparsamide) upon experimental infections of *Trypanosoma rhodesiense*..... 193
MEANS, J. H., BOCK, A. V., and WOODWELL, M. N. Studies of the acid-base equilibrium in disease from the point of view of blood gases..... 201
STEVENS, FRANKLIN A., BRADY, JOHN W. S., and WEST, RANDOLPH. Relation between the virulence of streptococci and hemolysin..... 223
LUND, CHARLES C., SHAW, LOUIS A., and DRINKER, CECIL K. Quantitative distribution of particulate material (manganese dioxide) administered intravenously to the dog, rabbit, guinea pig, rat, chicken, and turtle..... 231
NOGUCHI, HIDEYO, and KLIGLER, I. J. Experimental studies on yellow fever in northern Peru..... 239
NOGUCHI, HIDEYO, and KLIGLER, I. J. Immunology of the Peruvian strains of *Leptospira icteroides*..... 253
LEWIS, WARREN H., and WEBSTER, LESLIE T. Migration of lymphocytes in plasma cultures of human lymph nodes. Plates 10 and 11..... 261
FOOT, NATHAN CHANDLER. Studies on endothelial reactions. IV. The endothelium in experimental general miliary tuberculosis in rabbits. Plate 12..... 271
RICH, ARNOLD RICE. Condition of the capillaries in histamine shock. Plates 13 to 17..... 287

No. 3, MARCH 1, 1921.

MURPHY, JAMES B., HUSSEY, RAYMOND G., NAKAHARA, WARO, and STURM, ERNEST. Studies on x-ray effects. VI. Effect of the cellular reaction induced by x-rays on cancer grafts. Plates 18 to 20..... 299

MURPHY, JAMES B., HUSSEY, RAYMOND G., STURM, ERNEST, and NAKAHARA, WARO. Effect of induced cellular reaction on the fate of cancer grafts. IV. Studies on lymphoid activity. Plates 21 to 23..... 315

NAKAHARA, WARO, and MURPHY, JAMES B. The lymphocyte in natural and induced resistance to transplanted cancer. VI. Histological comparison of the lymphoid tissue of naturally immune and susceptible mice. Plates 24 to 26..... 327

HUSSEY, RAYMOND G. General leucocytic response of the guinea pig during the reaction of artificial immunity in experimental tuberculous infection..... 337

LEWIS, WARREN H., and WEBSTER, LESLIE T. Giant cells in cultures from human lymph nodes. Plates 27 to 31..... 349

OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. II. Filterability and resistance to glycerol. Plates 32 to 34..... 361

OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. III. Studies of the concurrent infections. Plate 35. 373

BLAKE, FRANCIS G., and TRASK, JAMES D., JR. Studies on measles. I. Susceptibility of monkeys to the virus of measles. Plate 36..... 385

BLAKE, FRANCIS G., and TRASK, JAMES D., JR. Studies on measles. II. Symptomatology and pathology in monkeys experimentally infected. Plates 37 to 41..... 413

No. 4, APRIL 1, 1921.

MURPHY, JAMES B., NAKAHARA, WARO, and STURM, ERNEST. Studies on lymphoid activity. V. Relation between the time and extent of lymphoid stimulation induced by physical agents and the degree of resistance to cancer in mice..... 423

NAKAHARA, WARO, and MURPHY, JAMES B. Studies on x-ray effects. VII. Effect of small doses of x-rays of low penetration on the resistance of mice to transplanted cancer..... 429

NAKAHARA, WARO, and MURPHY, JAMES B. Studies on x-ray effects. VIII. Influence of cancer inoculation on the lymphoid stimulation induced by small doses of x-rays..... 433

SMITH, THEOBALD. The etiological relation of *Bacillus actinoides* to bronchopneumonia in calves. Plates 42 to 50..... 441

ALEXANDER, HARRY L. Precipitin response in the blood of rabbits following subarachnoid injections of horse serum..... 471

LEWIS, MARGARET REED. Granules in the cells of chick embryos produced by egg albumin in the medium of tissue cultures. Plate 51..... 485

BROWN, WADE H., PEARCE, LOUISE, and WITHERBEE, WILLIAM D. Experimental syphilis in the rabbit. VI. Affections of bone, cartilage, tendons, and synovial membranes. Part 1. Lesions of the skeletal system. Plates 52 to 60..... 495

BROWN, WADE H., PEARCE, LOUISE, and WITHERBEE, WILLIAM D. Experimental syphilis in the rabbit. VI. Affections of bone, cartilage, tendons, and synovial membranes. Part 2. Clinical aspects of syphilis of the skeletal system. Affections of the facial and cranial bones and the bones of the forearm. Plates 61 to 66..... 515

BROWN, WADE H., PEARCE, LOUISE, and WITHERBEE, WILLIAM D. Experimental syphilis in the rabbit. VI. Affections of bone, cartilage, tendons, and synovial membranes. Part 3. Syphilis of the posterior extremities with other affections of a miscellaneous type. Plates 67 to 72..... 525

ELLERMANN, VILHELM. A new strain of transmissible leucemia in fowls (Strain H)..... 539

No. 5, MAY 1, 1921.

BROWN, WADE H., and PEARCE, LOUISE. Superinfection in experimental syphilis following the administration of subcurative doses of arsphenamine or neoarsphenamine. Plates 73 and 74..... 553

CHURCHMAN, JOHN W. Further studies on the behavior of bacteria toward gentian violet. Plates 75 to 77..... 569

CHURCHMAN, JOHN W., and KAHN, MORTON C. Communal activity of bacteria. Plates 78 to 80..... 583
BUNTING, C. H., and HUSTON, JOHN. Fate of the lymphocyte. 593
MACKENZIE, GEORGE M., and LEAKE, W. H. Relation of antibody and antigen to serum disease susceptibility..... 601
BLAKE, FRANCIS G., and TRASK, JAMES D., JR. Studies on measles. III. Acquired immunity following experimental measles..... 621
STADIE, WILLIAM C. Studies on blood changes in pneumococcus infections. An experimental study of the formation and fate of methemoglobin in the blood..... 627
EBELING, ALBERT H. Fibrin and serum as a culture medium.. 641
ADACHI, KIYOHISA. Flagellum of the microorganism of rat-bite fever. Plates 81 and 82..... 647
ANDERSON, RUTH A., and SCHULTZ, OSCAR T. Immunologic study of strains of *Bacillus pfeifferi* isolated from a case of meningitis..... 653
GRAYBILL, H. W. The incidence of blackhead and occurrence of *Heterakis papillosa* in a flock of artificially reared turkeys. 667

No. 6, JUNE 1, 1921.

DRINKER, CECIL K. A useful heart method. Plates 83 and 84. 675
BROWN, J. HOWARD. An improved anaerobe jar..... 677
COHN, ALFRED E., and NOGUCHI, HIDEYO. Etiology of yellow fever. XIII. Behavior of the heart in the experimental infection of guinea pigs and monkeys with *Leptospira icteroides* and *Leptospira icterohæmorrhagiæ*. Plates 85 and 86. 683
KOLMER, JOHN A., and SANDS, JOSEPH R. Chemotherapeutic studies with ethylhydrocupreine hydrochloride in experimental pneumococcus pleuritis. Plates 87 to 94..... 693
OLITSKY, PETER K., and GATES, FREDERICK L. Experimental studies of the nasopharyngeal secretions from influenza patients. IV. Anaerobic cultivation. Plates 95 to 99.... 713
MCMASTER, PHILIP D., and ROUS, PEYTON. The biliary obstruction required to produce jaundice..... 731

McJunkin, Frank A. Tuberculin hypersensitiveness in non-tuberculous guinea pigs induced by injections of bacillus-free filtrates..... 751
Thjötta, Theodor. Studies on bacterial nutrition. I. Growth of *Bacillus influenzæ* in hemoglobin-free media..... 763
De Kruif, Paul H. Dissociation of microbic species. I. Coexistence of individuals of different degrees of virulence in cultures of the bacillus of rabbit septicemia..... 773
Auer, John, and Witherbee, William D. Studies on decreasing the reaction of normal skin to destructive doses of x-rays by pharmacological means and on the mechanism involved. Plates 100 to 102..... 791
Murphy, James B., Witherbee, William D., Craig, Stuart L., Hussey, Raymond G., and Sturm, Ernest. Effect of small doses of x-rays on hypertrophied tonsils and other lymphoid structures of the nasopharynx. Plates 103 to 108..... 815
Index to Volume XXXIII..... 833

THE RETICULAR MATERIAL OF DEVELOPING BLOOD CELLS.

By EDMUND V. COWDRY, Ph.D.

(From the Anatomical Laboratory of the Peking Union Medical College, Peking, China.)

Plate 1.

(Received for publication, September 14, 1920.)

The object of this paper is to determine the appearance of the reticular material of normal blood cells with a view to preparing the way for further experimental studies, using it as an indicator of cell activity and cell injury. The extensive literature on the subject of this material has already been reviewed in detail by Holmgren (1901–02), Benda (1914), Duesberg (1914), and Cajal (1914). Very little has been done on the blood.

I have used the bone marrow of guinea pigs, applying the osmic acid method of Kopsch (1902),[1] the uranium and silver nitrate method of Cajal (1912),[2] and the methods of fixation recommended by Bensley (1910).[3] At the same time, the observations have been controlled by the examination of unstained and supravitally stained living cells by direct and oblique illumination.

Sinigaglia (1910) was apparently the first to discover a circumnuclear, net-like structure in the red blood cells of young amphibia, and von Berenberg-Gossler (1912)[4] has recorded a similar but eccentrically placed network in the blood cells of chick embryos. Fañanás (1912),[5] with the aid of Cajal's silver and uranium nitrate method, has attempted to determine the changes which this network undergoes during the development of red blood cells. He has found that with the appearance of hemoglobin it undergoes regressive changes, becoming condensed and finally disappearing completely, but he does not claim that there is any functional relation between its disappearance and the formation of hemoglobin.

[1] Kopsch (1902), p. 929.
[2] Cajal (1912), p. 211.
[3] Bensley (1910), p. 192.
[4] von Berenberg-Gossler (1912), p. 62.
[5] Fañanás (1912), p. 251.

Results Obtained with Erythroblasts.

Though I have been unable to detect any trace of the existence of reticular material in living red blood cells, I have produced a blackened network in the cytoplasm of erythroblasts by the methods of Kopsch and Cajal. Of the two methods the prolonged treatment with osmic acid, as advised by Kopsch, is in my opinion subject to the least criticism because it is relatively simple and does not distort the cells or destroy the specific granulations. Figs. 4 to 12 illustrate the irregularity and variability of the reticular material in erythroblasts.

Like von Berenberg-Gossler, I find that it is usually eccentrically placed and rarely if ever circumnuclear. In my preparations it is generally compact, varying, however, in size as well as in the coarseness of its meshes. Frequently in neighboring cells no trace of it can be seen. In the cells which show it there are usually some blackened granules of variable size. It almost seems as though stages in the formation of the network can be distinguished through the alignment and confluence of these granules, or that the granules are produced through the disintegration of the network. As the erythroblasts develop, the reticular material becomes smaller in amount and finally disappears in the mature erythrocytes. It is impossible to say how close is the parallelism between these changes and the formation of hemoglobin, because my preparations do not permit an accurate determination of the amount of hemoglobin.

Cajal's method brings to light a similar network, but unfortunately the preservation of the cells is so poor that it is often difficult even to identify them. Erythroblasts prepared by this method are shown in Figs. 14, 15, 18, and 20. In spite of their larger size and greater complexity, these networks resemble closely those in the Kopsch preparations described above. Similar blackened granules are also to be seen which perhaps bear the same relation to the network. Fig. 18 is suggestive in this connection because the blackened granules seem to be embedded in a more lightly colored material which forms the strands of the network.

The relation, however, between this blackened reticular material and the canalicular apparatus which may be demonstrated in the erythroblasts by fixation in a mixture of formalin and potassium

bichromate, as recommended by Bensley (1910), is not so clear. It is true that this system of clear canals is usually eccentrically placed to one side of the nucleus (Figs. 22, 23, and 25), that clear droplets suggestive of the blackened granules may often be discerned near it, and that it disappears as differentiation goes on, so that no trace of it can be found in mature erythrocytes (Fig. 28); but, on the other hand, the clear canals are more sharply defined (Fig. 27) and are often of greater girth (Fig. 30). They tend also to be more pointed and certainly look more like fixation artifacts, or better, greatly distorted pictures of some actually existing material, particularly when they are very pronounced as in Figs. 26, 31, and 36.

Although I have employed a large variety of mixtures and their individual ingredients in different concentrations, I have not been successful in attempts to determine what factor or factors in fixation are responsible for the appearance of these canals, which cannot be seen clearly in the living cell. It is difficult, if not impossible, to alter the caliber of the canals experimentally. Duesberg (1920)[6] postulates the existence of two distinct kinds of canalicular apparatus (trophospongium) one of which is identical with the reticular material (apparatus of Golgi) and the other is not. He writes as follows:

> "I came to the conclusion . . . . that two categories of cells should be distinguished: The neurones and non-nervous cells with a localized trophospongium on the one side, and the non-nervous cells with a diffuse trophospongium on the other. As to the latter, the identity of both formations can be rejected without further discussion; for, while the trophospongium extends all over the cytoplasm, the apparatus of Golgi is localized at one pole of the nucleus. As to the former, both formations appear to me identical."

The expression "identical" is one which I should hesitate to apply, as it cannot be interpreted to mean anything less than similarity in all respects. Obviously, the blackened apparatus of Golgi is not identical with a system of clear canals brought to light by a totally different method. One is a silver deposit, the other a portion of the cytoplasm devoid of silver which can only be stained with great difficulty. If we refer to two cells even of the same category or

[6] Duesberg (1920), p. 75.

to minute portions of them as being identical we are overlooking the fact that living material is constantly changing both qualitatively and quantitatively and that it is these changes which make life possible.

According to Duesberg's theory there are two kinds of trophospongium which are distinguished solely by the fact that the trophospongium is either localized or diffuse. There are no other differential characteristics. As a matter of fact, all the other known properties of the localized and diffuse trophospongium appear to be similar. Duesberg does not give sufficient consideration to the vagaries of technique. It is conceivable that the diffuse canals called trophospongium and the localized apparatus of Golgi, though quite different in appearance in fixed tissues, may have been produced through the action of diametrically opposed methods of technique upon somewhat similar material. Within the compass of a single cell whose cytoplasm is fluid and in more or less constant motion spacial differences are not of very great moment. A comparison of Figs. 22, 23, 25, 26, and 30 shows the remarkable variations in the topography of the trophospongium which may be seen in cells of the same type treated in the same way.

Results Obtained with Leucocytes.

Von Bergen (1904),[7] Kolster (1913), and others have alluded to the occurrence of reticular material in granular leucocytes. Here, as in the case of the erythroblasts, the methods of Kopsch and Cajal reveal what cannot be seen by the most painstaking observation of living cells. By the method of Kopsch a few scattered granules are blackened in the amphophils and eosinophils (Figs. 1 and 2), while nothing is brought to light in the basophils (Fig. 3). The lack of definite grouping on the part of these granules points to the conclusion that they may have nothing to do with the reticular material. Fortunately, Cajal's method gives more definite results (Figs. 13, 16, 17, 19, and 21). It will at once be remarked that the blackened networks are much smaller in proportion to the size of the cells than in erythroblasts. They are also more condensed, with closer meshes, and are

[7] von Bergen (1904), p. 542.

almost invariably situated in the concavity of the nucleus near the centrosome. Blackened granules, like those in the erythroblasts, are not apparent in the amphophilic leucocytes shown in Figs. 13, 16, and 17. This may mean that all the material has been used up in the formation of the networks.

Granular leucocytes in preparations made by fixation in formalin and potassium bichromate are depicted in Figs. 24, 29, and 32 to 35. In Fig. 24 the canals are shown in an amphophil. They are much larger than the blackened reticular material and are not restricted to the region of the nuclear concavity. In Fig. 29 they are just as large and conspicuous as in certain erythroblasts; *i.e.*, Figs. 26 and 30. Other myelocytes and leucocytes fail to show any trace of the canalicular apparatus (Figs. 32 to 35). Considerable variability is apparent even in adjacent cells in the same section.

Results Obtained with Lymphocytes.

I have not been successful in finding any trace of the presence of a reticular or canalicular apparatus in living cells of the lymphocytic series. In the Kopsch and Cajal preparations of the bone marrow it is only possible to distinguish the lymphoblasts from the erythroblasts through the absence of hemoglobin, and then not with certainty, the nuclear characteristics being indistinct. I have found, however, in common with other workers, that the lymphocytes inhabiting other tissues, such as the thyroid, show a very definite blackened network at one side of the nucleus when treated by Cajal's method. In this respect they resemble closely the erythroblast illustrated in Fig. 10.

DISCUSSION.

There can be no doubt that these observations indicate the existence of some material in the cytoplasm of developing red blood cells, leucocytes, and lymphocytes which reacts in a more or less characteristic way to two quite different methods of technique. Since investigators have also found that a large number of nerve cells, gland cells, and epithelial cells, in fact almost all the active cells of the body, react in much the same way when treated by these methods, this substance, or substances very closely related to it, must be widely

distributed in living protoplasm, and of fundamental importance. This material tends to exhibit the following properties. (1) Under certain conditions, which have not yet been fully determined, it may be blackened by treatment with osmic acid and silver nitrate. It may also be frequently stained with resorcin-fuchsin after fixation in trichloracetic acid. Though I have obtained satisfactory preparations by this method in other tissues (brain, pancreas, etc.) the blood cells have thus far proved refractory. (2) The material is arranged in a network of anastomosing strands, the form of which is to some extent characteristic of different cell types. (3) This network is broken up on cell division and is distributed in approximately equal amounts to the two daughter cells. It undergoes definite and progressive changes during cell differentiation. (4) In secretory cells with pronounced polarity, such as the acinus cells of the pancreas, it is invariably found in the region of the cytoplasm between the nucleus and the distal border. When the material is distributed in the form of a circumnuclear network it usually occupies an intermediate position, leaving a zone of clear cytoplasm about the nucleus and beneath the cell wall. (5) It is often, though not always, found in close association with the centrosome which is presumably a center of unusually high or low cytoplasmic activity.

Following the discovery by Golgi of reticular material in nerve cells, claims were made, which have not since been substantiated, that this material is composed of easily recognizable cytoplasmic constituents like the mitochondria and the chromidial substance. Recent work, however, tends to show that it is a constituent of the ground substance which in living cells appears to be homogeneous when viewed with the best apochromatic lenses. This homogeneity is, of course, apparent, not real, for true homogeneity would be incompatible with chemical activity. Suitable methods for the detection of this reticular material in living cells are at present lacking. We have no reason to suppose that the form of the reticular material in living protoplasm is very different from that seen in fixed preparations. It is probable, however, that in the living condition its outlines are less distinct and that it merges gradually into the remainder of the cytoplasm. With fixation a certain amount of condensation and shrinkage occurs so that this reticular material would

occupy a rather larger area of the cytoplasm in the living cell than in fixed preparations. The phenomena of ameboid motion, phagocytosis, and the freedom of motion of certain intracellular granules are incompatible with the existence of a rigid network within the cell. Moreover, M. R. Lewis and W. H. Lewis (1914–15) have repeatedly studied cultures of living connective tissue cells, yet they have never recorded the presence of a semisolid network of material, although Deineka (1912)[8] experienced no difficulty in bringing a network to light in connective tissue cells by his silver method. It is interesting also to note that Chambers (1915) has actually dissected cells in which the existence of this network of material has been demonstrated by the methods of Kopsch and Cajal without remarking upon the occurrence of any kind of framework of resistant nature. All the information at hand suggests that, though the material is restricted to certain cytoplasmic areas, it is nevertheless of watery consistency, being even more fluid than the general ground substance, and is probably changing its form continually as it plays its obscure part in the activities of the cell, for no two networks as seen in fixed preparations of blood cells, or any other kind of cell, are exactly alike.

If this idea regarding the fluidity of the material is correct we must be dealing with a system of intracellular spaces or canals as claimed by Bensley. The walls of the canals appear to be extremely thin. It has never been possible to stain them in fixed preparations. In all probability they are merely surfaces of contact between fluids of different character. Slight changes in the reticular material or in the surrounding cytoplasm would certainly modify the visibility of the surfaces. It seems to me also that in living protoplasm the reticular material may to some extent intermingle with the surrounding cytoplasm instead of being sharply separated from it. Since its refractive index is apparently the same as that of the cytoplasm it could not be expected to be visible in the living cell until selective methods for its coloration have been devised. Bensley (1911–12),[9] however, has been able to observe a system of clear canals in islet cells of the pancreas stained supravitally with neutral red. The canals show up as clear spaces in a cytoplasm otherwise crowded with

[8] Deineka (1912), pp. 303, 306.

[9] Bensley (1911–12), p. 365.

granules. On one occasion I observed a similar phenomenon in nerve cells (1912–13)[10] but all my efforts to discern this reticular material in blood cells and in other cells of the body have thus far been unavailing. In both these cases some time elapsed between the removal of the cells from the animal and the detection of the canals. Their appearance may, therefore, be due, as already intimated, to some change in the reticular material or in the cytoplasm, or in both. The fluidity and lability of the material would make it particularly susceptible to the change in environment.

Though the question of terminology is a minor one the least criticism will be caused if this peculiar substance, or mixture of substances, is called reticular material, instead of reticular apparatus, until more definite information is obtained about it. The idea of a distinct system of canals is not sustained, and speaking of it as a cell organ suggests that it is a more or less complex unit built up of specialized components each with a definite function, like the constituent tissues of the bodily organs, in favor of which there is as yet insufficient evidence. Study from the standpoint of microchemistry would throw further light on this subject.

The wide distribution of this reticular material in the three categories of blood cells as well as in active cells of almost all kinds, and the reasonableness of Bensley's (1910)[11] hypothesis that the canalicular apparatus is "the physiologic and morphologic equivalent of the vacuolar system of the plant cell," make it apparent that this is a cytoplasmic constituent of great importance.

SUMMARY.

Erythroblasts, leucocytes, and lymphocytes resemble other cells of the body in containing a restricted area of fluidity in their cytoplasm. In special preparations this fluid appears in the form of a more or less complicated reticulum which seems to be continually but slowly changing in shape. For convenience the fluid may provisionally be referred to as reticular material, emphasis being laid on its composition rather than its form. As a working hypothesis it is safe to

[10] Cowdry (1912–13), p. 490.
[11] Bensley (1910), p. 193.

assume that the chemical and physical properties of this material vary in cells of different kinds as well as in different stages in the activity of the same cell. The conclusion that it is "identical" in different cells, because the present crude methods of technique reveal no fundamental differences, would be as incorrect as the statement that the serum of different animals is identical, because no difference is observed on microscopic examination.

BIBLIOGRAPHY.

Benda, C., Die Bedeutung der Zelleibstruktur für die Pathologie, I. Die Morphologie der Zelle, *Verhandl. deutsch. path. Ges.*, 1914, xvii, 5.

Bensley, R. R., On the nature of the canalicular apparatus of animal cells, *Biol. Bull.*, 1910, xix, 179; Studies on the pancreas of the guinea pig, *Am. J. Anat.*, 1911–12, xii, 297.

von Berenberg-Gossler, H., Die Urgeschlestszellen des Hühnerembryos am 3. und 4. Bebrütungstage, mit besonderer Berücksichtigung der Kern- und Plasmastrukturen, *Arch. mikr. Anat., 2te Abt.*, 1912, lxxxi, 24.

von Bergen, F., Zur Kenntnis gewisser Strukturbilder (Netzapparate, Saftkanälchen, Trophospongien) im Protoplasma verschiedener Zellenarten, *Arch. mikr. Anat.*, 1904, lxiv, 498.

Cajal, S. R., Fórmula de fijación para la demonstración fácil del aparato reticular de Golgi y apuntes sobre la disposición de dicho aparato en la retina, en los nervios y algunos estados patológicos, *Trab. lab. invest. biol. Univ. Madrid*, 1912, x, 211; Algunas variaciones fisiológicas y pathológicas del aparato reticular de Golgi, 1914, xii, 127.

Chambers, R., Jr., Microdissection studies on the physical properties of protoplasm, *Lancet-Clinic*, 1915, cxiii, 363.

Cowdry, E. V., The relations of mitochondria and other cytoplasmic constituents in spinal ganglion cells of the pigeon, *Internat. Monatschr. Anat. u. Physiol.*, 1912–13, xxix, 473.

Deineka, D., Der Netzapparat von Golgi in einigen Epithel- und Bindegewebszellen während der Ruhe und während der Teilung derselben, *Anat. Anz.*, 1912, xli, 289.

Duesberg, J., Trophospongien und Golgischer Binnenapparat, *Anat. Anz.*, 1914, xlvi, suppl., 11; Cytoplasmic structures in the seminal epithelium of the opossum, *Carnegie Inst. Washington, Pub. No. 272*, 1920, Contributions to embryology, ix, 47.

Fañanás, J. R., Nota preventiva sobre el aparato reticular de Golgi en el embrión de pello, *Trab. lab. invest. biol. Univ. Madrid*, 1912, x, 247.

Holmgren, E., Neue Beiträge zur Morphologie der Zelle, *Ergebn. Anat. u. Entwklngsgesch.*, 1901–02, xi, 274.

Kolster, R., Ueber die durch Golgis Arsenik- und Cajals Urannitrat-Silbermethode darstellbaren Zellstrukturen, *Anat. Anz.*, 1913, xliv, suppl., 124.

Kopsch, F., Die Darstellung des Binnennetzes in spinalen Ganglienzellen und anderen Korperzellen mittels Osmiumsaure, *Sitzungsber. k. Akad. Wissensch. Math.- naturw. Cl., Wien.*, 1902, xl, 929.

Lewis, M. R., and Lewis, W. H., Mitochondria (and other cytoplasmic structures) in tissue cultures, *Am. J. Anat.*, 1914–15, xvii, 339.

Sinigaglia, G., Osservazioni sulla struttura dei globuli rossi, *Arch. sc. med.*, 1910, xxxiv, 191.

EXPLANATION OF PLATE 1.

All the figures have been drawn with a Zeiss objective 1.5 mm., compensating ocular 8, and camera lucida. Magnification, × 2,200.

FIGS. 1 to 12. Cells from the bone marrow of an adult male guinea pig fixed in 2 per cent osmic acid for 15 days for the demonstration of the reticular apparatus after the method of Kopsch.

FIGS. 13 to 21. Cells from the bone marrow of a male guinea pig of 240 gm. prepared by the uranium nitrate method of Cajal with slight modifications.

FIGS. 22 to 36. Cells from the bone marrow of the same guinea pig fixed in Kopsch's formalin-bichromate mixture and stained with fuchsin and methyl green.

FIG. 1. Amphophil leucocyte without any trace of a blackened network. A few granules are present which are blackened with osmic acid; the others are of a dull gray tone.

FIG. 2. Eosinophil leucocyte with no indication of a blackened network. It contains a series of smaller granules which are intensely blackened and other larger spherical ones of a gray color.

FIG. 3. Basophil leucocyte without either network or blackened granules.

FIG. 4. Large erythroblast with blackened granules exhibiting a tendency toward network formation.

FIG. 5. Erythroblast with a rather more definite network at one side of the nucleus.

FIG. 6. Erythroblast containing only three spherical blackened granules of variable size.

FIG. 7. Erythroblast with granules of more irregular shape.

FIG. 8. Erythroblast with apparently clear cytoplasm.

FIG. 9. Erythroblast with an almost completely blackened network, the individual strands of which may be followed by careful focusing. The cytoplasm contains also some blackened granules.

FIG. 10. Erythroblast with a blackened network of much closer mesh. No blackened granules are to be seen in the cytoplasm.

FIG. 11. Erythroblast with a large open network and cytoplasmic granules.

FIG. 12. Erythroblast with a blackened network of which the strands are noticeably more slender.

FIG. 13. Polymorphonuclear amphophil leucocyte with several large blackened granules in the concavity of the nucleus in the region where the centrosome is located.

FIG. 14. Large erythroblast with a complete network at one pole of the nucleus.

FIG. 15. Large erythroblast with an extensive and very intricate network and some cytoplasmic granulation.

FIG. 16. Polymorphonuclear amphophil leucocyte with a very simple network in the concavity of the nucleus.

FIG. 17. Polymorphonuclear amphophil leucocyte with slightly more complicated network in the same location.

FIG. 18. Large cell, possibly an erythroblast, showing the formation of a network by the alignment of discrete granules.

FIG. 19. Amphophil myelocyte with network formation in the nuclear concavity.

FIG. 20. Erythroblast with very circumscribed, closely meshed network.

FIG. 21. Coarsely granular leucocyte, probably eosinophilic, showing likewise network formation in the concavity of the nucleus.

FIG. 22. Erythroblast containing three irregular clear spaces (negative demonstration).

FIG. 23. Erythroblast with the clear canals forming a complicated coil.

FIG. 24. Amphophil polymorphonuclear leucocyte with clear canals in the peripheral cytoplasm as well as in the concavity of the nucleus.

FIG. 25. Erythroblast with clear canals and vacuoles distributed throughout the entire cytoplasmic area.

FIG. 26. Large cell, probably also an erythroblast, showing the extreme of canalicular and vacuolar formation as well as some crenation. The canals and vacuoles are of large caliber.

FIG. 27. Erythroblast containing clear canals of very fine diameter.

FIG. 28. Erythrocyte devoid of any trace of canalicular formation.

FIG. 29. Large myelocyte containing a very complicated system of clear canals.

FIG. 30. Erythroblast with canals of maximum caliber. The forked ones are especially characteristic.

FIG. 31. Erythroblast containing a peculiar clear circle.

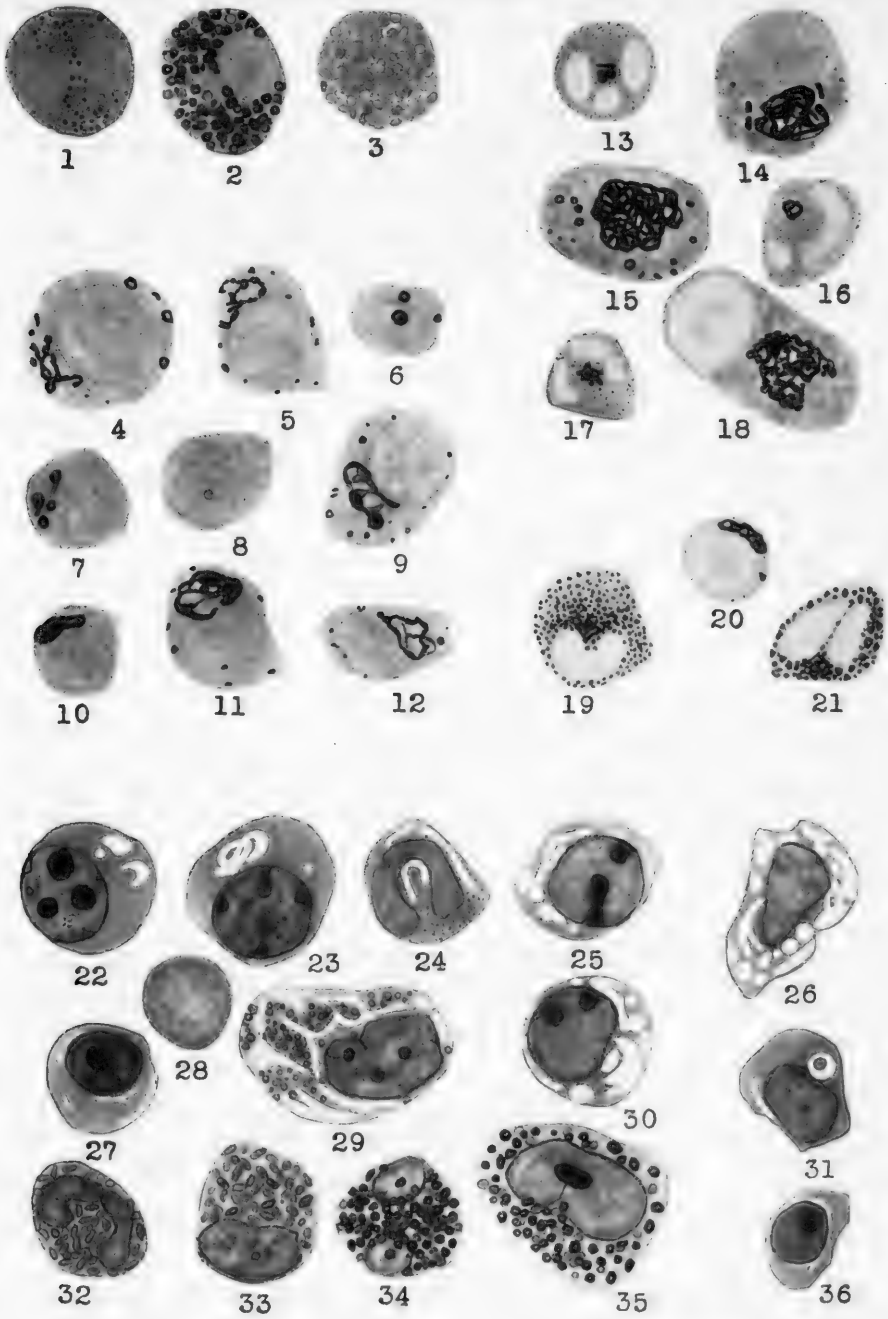
FIG. 32. Basophil polymorphonuclear leucocyte without any indication of canalicular formation.

FIG. 33. Basophil myelocyte likewise without visible canalicular apparatus.

FIG. 34. Eosinophil leucocyte in the same condition.

FIG. 35. Eosinophil myelocyte without any sign of canalicular apparatus.

FIG. 36. Erythroblast with clear clefts almost surrounding the nucleus.



(Cowdry: Developing blood cells.)

STREPTOCOCCI OCCURRING IN SOUR MILK.

By F. S. JONES, V.M.D.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

(Received for publication, June 24, 1920.)

Considerable confusion seems to exist regarding the specific cultural characters of the group of organisms which sour milk. Many have noted the presence of enormous numbers of streptococcic forms in sour milk. The morphology of such bacteria has been studied in detail by Grotenfeld,[1] Günther and Thierfelder,[2] Kruse,[3] and others. Kruse has given the name of *Streptococcus lacticus* to the organism most frequently found and called attention to its resemblance to the pneumococcus. Many, however, disagree with Kruse and place it in the genus *Bacterium*; such names as *Bacterium lacticus*, *Bacterium güntheri*, and *Bacillus lactis acidi* are frequently met with.

In addition to the confusion in nomenclature, one frequently finds reference to the occurrence of *Streptococcus lacticus* in the udder, feces, and saliva, or on the skin of cows. Furthermore, many believe that the milk-souring type is indistinguishable in most respects from the pyogenic type.

Ruediger[4] studied streptococci isolated from milk freshly drawn into milk pails and sterile bottles. Of 32 strains grown in blood agar plates, 8 produced considerable hemolysis about the colonies, 14 were faintly hemolytic, 9 produced green zones, and 1 was non-hemolytic. All the organisms were cocci arranged in chains; rod-like elements were not observed. Ruediger considered that the eight hemolyzing strains were *Streptococcus pyogenes* and the other faintly hemolyzing and green-producing organisms belonged to the *Streptococcus lacticus* group. Further evidence to warrant this assumption is not submitted.

Heinemann[5] states that the group comprises two species,—a bacterium, *B. aerogenes* var. *lacticus* and *Streptococcus lacticus*. It is stated that *Streptococcus lacticus* agrees in morphological and cultural characters with the human pathogenic, sewage and fecal streptococci. He believes that the milk-souring streptococci can be detected on the external surfaces and in the feces of cows as well as

[1] Grotenfeld, G., *Fortschr. Med.*, 1889, vii, 121.

[2] Günther, C., and Thierfelder, H., *Arch. Hyg.*, 1895, xxv, 164.

[3] Kruse, *Centr. Bakt., 1te Abt., Orig.*, 1903, xxxiv, 737.

[4] Ruediger, G. F., *Am. J. Pub. Health*, 1912, ii, 107.

[5] Heinemann, P. G., *J. Infect. Dis.*, 1906, iii, 173.

in the milk during all stages of handling. In a later paper Heinemann[6] published the results of some experiments with two strains of streptococci, one isolated from certified milk, the other from ice-cream. By the passage of these cultures through a series of rabbits and guinea pigs their virulence was increased. In addition, the organisms grew feebly. They produced much less acid in dextrose and lost the power to ferment saccharose, mannitol, and salicin.

Conn, Esten, and Stocking[7] described *Streptococcus lacticus* as a short chained, Gram-positive coccus, which grew on agar as an exceedingly delicate film. Gelatin colonies were characteristic but the medium was not liquefied. Dextrose, lactose, and saccharose were fermented without gas formation. Milk was coagulated. They found these organisms in great numbers in sour milk and state that they frequently made up 99 per cent of the flora.

Shippen[8] in studying the microorganisms of milk in Baltimore referred to *Streptococcus lacticus* as *Bacterium güntheri*. It was the most common type observed by him in sour milk but its presence was also noted in fresh (unsoured) milk. Like Kruse, he stated that the forms varied from short slender rods to distinct coccus-like forms. Many strains closely resembled streptococci when grown in liquid media, but search always revealed some rod-like forms. In these cultures the elongated elements predominated but cocci could also be found. He considers that the sour milk organisms differ from *Streptococcus pyogenes* in several respects; namely, in form, in their inability to hemolyze, their ability to grow at lower temperatures, their lack of pathogenicity for laboratory animals, and their high thermal death-point (65–70°C.).

McGuire[9] considers that *Streptococcus lacticus* is a normal inhabitant of cow feces. Apparently the species was identified largely by morphology and the points noted by Shippen.

Weigmann[10] divides the milk streptococci into two groups: the *Streptococcus mastitidis* Guillebeau group characterized by the coagulation of milk and the formation of gas from carbohydrates; and the lactic acid group, *Streptococcus lacticus*, or *Streptococcus güntheri*. The latter class fails to produce gas. It is surprising to note the frequency with which the mastitis streptococci are referred to as gas-producing organisms throughout the literature.

Sherman and Albus[11] have shown that udder streptococci differ from those found in sour milk. They isolated and studied 50 strains from sour milk and as

---

[6] Heinemann, P. G., *J. Infect. Dis.*, 1915, xvi, 221.

[7] Conn, H. W., Esten, W. M., and Stocking, W. A., *Rep. Storrs Agric. Exp. Station*, 1906, 91.

[8] Shippen, L. P., *Bull. Johns Hopkins Hosp.*, 1914, xxv, 122.

[9] McGuire, P. F., *Bull. Johns Hopkins Hosp.*, 1915, xxvi, 386.

[10] Weigmann, H., in Sommerfeld, P., Handbuch der Milchkunde, Wiesbaden, 1909, 328.

[11] Sherman, J. M., and Albus, W. R., *J. Bact.*, 1918, iii, 153.

many from milk drawn directly from the udder into sterile bottles. The two classes of organisms were studied in groups. It was found that the individual elements of the sour milk type were elongated and grouped most frequently in pairs, but chain formation was common. The udder streptococci were spherical and grew as chains. The action of the two groups in the various media was noted. Thus 40 per cent of the lactic acid cocci fermented mannitol and a like proportion salicin. Of the udder type 76 per cent fermented saccharose and 16 per cent produced acid in salicin; all failed to ferment mannitol. They found that the milk-souring organisms were able to multiply at a temperature as low as 10°C. The udder streptococci grew better at 38°C. but 82 per cent were able to grow at 43°C. Considerable stress is laid on the reducing properties of the lactic acid organisms. The lactic acid types were able to grow in milk containing methylene blue and other dyes and were able to reduce the substances. The udder streptococci failed to grow in the methylene blue medium. In milk containing litmus members of both groups reduced the substance, but the lactic acid streptococci reduced litmus before coagulation, the others after the medium had coagulated.

It will be noted from the foregoing brief résumé that the specific identity of the so called *Streptococcus lacticus* is more or less obscure. The work of Sherman and Albus did much to show that differences existed between the lactic acid and udder groups.

It was considered advisable to study the species or groups of species with the idea that there exists a clear-cut differentiation from the udder or mastitis streptococci. With this in view, milk from various sources was permitted to sour at room temperature and in the incubator. Plate cultures were made from the milk at various times. The isolated organisms were studied in the same type of medium employed in the study of mastitis and equine streptococci.

EXPERIMENTAL.

The following experiment may be regarded as a type. Bottled market milk was purchased and after mixing distributed into sterile wide mouth bottles. These were permitted to stand at room temperature. The milk was examined at various intervals; usually an unopened bottle was chosen each time. A sample of the fresh milk was plated at once; subcultures were made from colonies resembling streptococci. In Table I the protocols of five samples of milk are given.

TABLE I.

Sample.	Length of time left at room temperature.	Acidity of whole milk.	Acidity of whey.		No. of organisms per cc.	Proportion of streptococci.	Subcultures referred to in Table II.
			Titration.	Hydrogen ion concentration.			
	days	*per cent*	*per cent*	*pH*			
A	0	1.3			2,160	5 per cent.	1, hemolytic. 3, " 4 A, non-hemolytic.
A	1	1.5			6,300,000	5 " "	7, hemolytic.
A	2	7.5	6.0	4.7	220,000,000	90 " " 2 per cent hemolytic.	13, " 15, non-hemolytic.
A	3	9.2	7.3	4.6	107,000,000	90 per cent.	16, " 18, " 18 A; "
A	4	8.4	6.9	4.4	720,000,000	90 " " 1 per cent hemolytic.	20, hemolytic. 21, non-hemolytic. 21 A, "
A	7	9.0	7.2	4.7	760,000,000	90 per cent.	23, "
A	14	8.1	7.4	4.8	960,000,000	85 " "	25, " 25 A, "
B	0				7,500	5 per cent hemolytic.	27, hemolytic.
B	1	5.3	4.2	5.3	12,800,000	90 per cent. 1 per cent hemolytic.	28, " 29 A, non-hemolytic.
B	2	9.1	6.8	4.8	3,500,000,000	95 per cent. Few hemolytic colonies.	30, " 30 A, "

B	3	9.6	7.7	4.6	5,000,000,000	90 per cent non-hemolytic.	31, non-hemolytic. 32 A, "
B	5	7.5	7.0	4.9	3,000,000,000	90 " " "	33, " 34, hemolytic.
						Less than 1 per cent hemolytic.	
B	7	9.0	8.6	4.8	1,500,000,000	95 per cent non-hemolytic. 1 " " hemolytic.	35, non-hemolytic. 36, hemolytic.
C	2					95 " " non-hemolytic.	26, non-hemolytic. 26 A, "
D	2 (incubator).	8.0	7.9	4.7	3,000,000,000	90 " " "	45, "
E	2 (room).	8.2	7.6	4.9	500,000,000	50 " " "	43, "

It will be noted from the table that Sample A was milk of high quality. It soured slowly, but between the 24th and 48th hours there appeared in the plates enormous numbers of non-hemolytic streptococci. These organisms practically overwhelmed all others and persisted throughout the observation. The hemolytic (mastitis) streptococci which were in the unsoured sample persisted until the 4th day. The non-hemolytic udder type was still found after 3 days of souring. Perhaps both types survived throughout, but could not be detected on account of the great number of other organisms. Sample B reveals about the same condition. The coagulation was more rapid and due to the growth of enormous numbers of non-hemolytic streptococci. The hemolytic streptococci observed in the fresh sample persisted for the 7 days allotted to the observation. They were unable to multiply with the same facility as the others. In Samples C, D, and E the non-hemolytic organisms also predominated.

It soon became apparent that souring could not be attributed to the common type of streptococci met with in udder and bottled milk. The udder types were able to persist through at least part of the souring period but not in sufficient numbers to be the cause of the phenomenon.

The detailed fermentative characters of the various strains noted in the protocols are given in Table II. It must be recognized that these cultures are merely types. Others have been studied but fail to show further differences from those recorded.

The figures given in Tables II and III represent the total acidity reached in the various tubes. All the fermented bouillon was of the same lot. Duplicate series were inoculated and incubated at 38°C. and at room temperature. The recorded figures represent the maximum acid production at the temperature best adapted for growth. Thus, Cultures 1, 3, 4 A, 7, 13, 18 A, and 20 grew better and produced more acid at 38°C., therefore the acid produced by them at this temperature has been recorded. On the other hand, all the other organisms adapted themselves better to temperatures of 20–22°C., so that the acid production has been given after incubation at this temperature.

TABLE II.

Fermentative Characters of Streptococci Noted in Table I.

Culture No.	Production of acid in.								
	Dextrose.	Lactose.	Saccharose.	Maltose.	Raffinose.	Inulin.	Mannitol.	Salicin.	Hemolysis.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
1	5.6	4.7	4.7	5.2	1.0	1.0	1.0	1.0	+
3	3.8	3.3	2.6	3.6	1.1	1.0	1.1	1.0	+
4 A	5.1	4.1	5.6	4.8	1.1	1.0	1.1	3.6	−
7	5.0	4.2	4.7	4.6	1.0	1.0	1.0	1.0	+
13	5.6	4.9	4.6	5.2	1.1	1.1	1.1	1.1	+
18 A	4.9	4.4	4.5	4.4	1.1	1.1	1.1	2.3	−
20	4.6	4.4	4.7	3.6	1.0	1.1	1.1	1.0	+
18	6.9	6.0	1.1	6.9	1.1	1.0	4.6	4.4	−
21	6.7	3.8	1.1	5.8	1.0	1.0	5.8	5.8	−
25 A	6.7	5.4	1.1	6.1	1.1	1.1	4.8	4.6	−
26	6.6	5.8	1.1	5.2	1.1	1.0	4.2	4.0	−
26 A	6.8	5.4	1.1	6.6	1.1	1.1	5.9	4.5	−
30	6.9	5.9	1.1	6.5	1.1	1.1	4.8	5.0	−
31	6.7	5.7	1.1	5.9	1.1	1.1	3.5	3.8	−
32 A	6.6	5.8	1.1	6.1	1.0	1.1	6.5	6.5	−
33	6.7	6.1	1.1	5.6	1.1	1.1	4.1	5.0	−
35	6.8	6.2	1.1	5.8	1.1	1.0	3.6	5.3	−
43	6.5	6.1	1.1	6.0	1.1	1.1	4.4	5.0	−
45	6.8	6.1	1.1	5.9	1.1	1.1	4.9	5.0	−
15	6.5	6.1	6.4	5.4	1.0	1.1	4.8	4.8	−
16	7.1	5.7	5.3	4.7	1.1	1.1	3.9	4.6	−
23	6.8	6.2	6.4	5.6	1.0	1.1	4.3	5.8	−
29 A	6.1	5.0	5.4	4.0	1.1	1.1	3.4	5.0	−
21 A	6.5	4.8	6.1	6.5	1.1	1.1	1.2	6.5	−
25	7.0	6.4	6.6	5.8	1.0	1.1	1.1	5.8	−
30 A	6.0	4.4	4.9	5.2	1.0	1.1	1.1	5.4	−
27	6.6	5.0	1.1	5.1	1.0	1.1	1.0	5.4	+
28	6.7	5.2	1.1	5.3	1.1	1.0	1.0	5.4	+
34	7.0	5.2	1.1	5.2	1.0	1.0	1.0	6.0	+
36	6.5	5.0	1.1	5.0	1.1	1.0	1.1	5.4	+

It will be noted that Cultures 1, 7, 13, and 20 are hemolytic streptococci which were found in both fresh and sour milk. These strains are identical with those associated with mastitis. They are aggluti-

nated by their specific group serum. Culture 3 is similar to the low acid-producing streptococci found in the udder and in market milk. This species has been discussed in detail in a previous communication.[12] Cultures 4 A and 18 A are characteristic non-hemolytic mastitis streptococci which agglutinate with their specific antiserum. All these cultures promptly coagulate milk at 38°C. Litmus was not, as a rule, reduced even after coagulation. Where reduction occurred it was usually confined to the bottom of the tube. Since these well recognized udder and mastitis streptococci were unable to multiply to any great degree in soured milk, it is assumed that such is not their function. In other words, their true habitat is within the udder.

In contradistinction to the preceding group, a second still larger series, comprising Strains 18, 21, 25 A, 26, 26 A, 30, 31, 32 A, 33, 35, 43, and 45, made up the bulk of the flora of clotted milk. These organisms differed in morphology from the mastitis types. In bouillon the elements were usually elongated and arranged in pairs and short chains. Spherical forms were also observed. The mastitis cultures produced chains of cocci. The second group of organisms attacked dextrose, lactose, maltose, mannitol, and salicin, but failed to ferment saccharose, raffinose, and inulin. They produced more acid in dextrose than did the mastitis streptococci. Litmus was reduced before milk containing it was coagulated.

The next four strains (Nos. 15, 16, 23, and 29 A) were isolated from two samples only. They, too, differed from the mastitis streptococci morphologically. They produced large amounts of acid in media containing dextrose, lactose, saccharose, maltose, mannitol, and salicin. Litmus was reduced.

Cultures 21 A, 25, and 30 A also produced large amounts of acid. They attacked the same substances as the non-hemolytic mastitis streptococci but produced more acid. Litmus was reduced by these cultures. The characteristic rod-like and coccoid elements occurring in pairs and short chains are a further point in their differentiation. They grew best and produced a maximum acidity at room temperature. In addition they were not agglutinated by mastitis streptococcus serum.

[12] Jones, F. S., *J. Exp. Med.*, 1920, xxxi, 347.

The hemolytic streptococci (Nos. 27, 28, 34, and 36) which persisted throughout the souring of Sample B differed from the usual udder streptococci in several characters. Morphologically they

TABLE III.

Comparative Acid Production by Streptococci in 1 Per Cent Fermented Broth at Incubator and Room Temperature.

Culture No.	Incubator temperature.		Room temperature.	
	Titration.	Hydrogen ion concentration.	Titration.	Hydrogen ion concentration.
	per cent	*pH*	*per cent*	*pH*
1	5.6	4.8	5.0	4.9
4 A	5.3	4.9	4.6	4.9
7	5.0	4.7	5.1	4.9
13	5.8	4.8	4.8	4.8
18 A	4.9	4.7	4.4	4.9
20	5.6	4.7	4.8	4.9
18	6.0	4.7	6.9	4.6
21	5.8	4.8	6.3	4.4
25 A	5.3	4.7	6.7	4.5
26	5.0	4.8	6.6	4.6
26 A	5.4	4.7	6.8	4.6
30	5.9	4.8	6.9	4.6
31	5.2	4.8	6.7	4.6
32 A	5.1	4.9	6.6	4.6
33	5.4	5.0	6.7	4.5
35	5.6	4.9	7.0	4.5
43	6.0	4.6	6.5	4.5
45	5.7	4.8	7.0	4.4
15	5.8	4.8	6.9	4.6
16	7.0	4.6	7.1	4.5
23	5.6	4.9	6.8	4.5
29 A	4.4	4.9	6.1	4.5
21 A	6.0	4.7	6.5	4.5
25	5.9	4.7	7.0	4.5
30 A	4.4	4.8	6.0	4.6
27	7.0	4.5	6.9	4.5
28	7.0	4.7	6.7	4.5
34	7.1	4.9	6.9	4.5
36	7.1	4.7	7.0	4.4

resembled the sour milk types. They did not reduce litmus completely and failed to ferment saccharose. They grew as well at 38°C. as at room temperature.

It has been asserted that the sour milk organisms grew better and produced more acid at room temperature. Experimental evidence that this is the case is offered in Table III. Duplicate inoculations into fermented bouillon of the same lot containing 1 per cent dextrose were made; one set was incubated at 38°C., the other at room temperature. Titrations and hydrogen ion concentration readings were made after 7 days. All cultures grew well at both temperatures. The results are given in Table III.

The figures indicate that cultures of the mastitis type (Nos. 1, 4 A, 7, 13, 18 A, and 20) produced more acid when grown in the incubator. The reverse is true for the others except for the hemolytic strains (Nos. 27, 28, 34, and 36). That the true milk-souring streptococci grow better at ordinary temperatures (20–22°C.) certainly points to other sources of origin than the saliva, feces, or udder of cows. Cultures 27, 28, 34, and 36 differed from both groups in many characters. They may be saprophytic forms which have gained entrance into the udder and are still adapting themselves to a new environment.

The pathogenic properties of the sour milk streptococci have been studied by so many that further experimental evidence seemed unnecessary. Many have reported the entire lack of pathogenesis of the members of this group for laboratory animals. Unfortunately such findings cannot be regarded as a method of differentiation between mastitis and sour milk streptococci, since the mastitis streptococci are also lacking in virulence for species other than bovines.

DISCUSSION.

From the preceding observations it seems clearly established that the lactic acid group consists of at least three species of organisms. Each species possesses characters which differentiate it from the streptococci which are associated with mastitis and the low acid-producing udder forms.

Sufficient data to warrant the assertion that *Streptococcus lacticus* is an udder inhabitant have not been brought forth. Sherman and Albus first showed conclusively that there existed distinct group differences between the udder and the milk-souring streptococci. Aside from the specific cultural differences here reported a number of other points throw light on the question. The milk-souring types

have not been met with in milk drawn directly from the udder. Such organisms have not been found in the fresh bottled milk from a dairy where a great many examinations of udder milk have been made. Mastitis and udder streptococci are usually present in this bottled milk. The milk-souring group grows best and produces more acid at a temperature of 20–22°C. The udder streptococci prefer a higher temperature (38°C.).

Rogers and Dahlberg[13] found in milk in a few instances non-saccharose-fermenting streptococci which attack mannitol. They also studied over 50 strains of udder origin. Among these but one attacked mannitol without acidulating media containing saccharose. This organism liquefied gelatin.

Many have pointed to several possible sources of origin. The saliva, feces, and skin of cows have been mentioned particularly. The specific identifications were usually made on morphological findings. Many of the streptococci from the saliva, skin, and feces do resemble the lactic acid organisms in form, but they differ in other essentials. The writer has isolated 35 strains of streptococci from the saliva of cows. Although mannitol fermentation occurred in one-third of the cultures, every strain fermented saccharose. In addition the amount of titratable acid produced in dextrose rarely exceeded 4 per cent. The bovine fecal streptococci were characterized by their ability to produce large amounts of acid in dextrose, lactose, saccharose, maltose, raffinose, inulin, and salicin. Mannitol was not fermented. Streptococci from the skin produced acid in fermented broth containing raffinose, saccharose, and mannitol in addition to dextrose, lactose, maltose, and salicin. One feels justified in asserting that if *Streptococcus lacticus* inhabits these regions it exists in such small numbers that it cannot be detected. The souring of milk cannot, therefore, be attributed to the usual types found in the saliva, feces, or vagina or on the skin of cows.

Sufficient evidence exists to rule out the udder or mastitis group as the usual cause of souring under natural conditions. These organisms are readily detected in bottled milk, but cannot be found in plate cultures prepared from milk soured for 3 or 4 days. When

[13] Rogers, L. A., and Dahlberg, A. O., *J. Agric. Research*, 1913–14, i, 491.

equal amounts of cultures of lactic acid and udder streptococci were added to tubes of sterile milk, the udder streptococci multiplied for 24 hours. Plates prepared from such milk after 48 hours at room or incubator temperature reveal only the lactic acid type. Under natural conditions probably other bacteria so alter the environment that the udder group is able to survive but not to multiply to any considerable degree.

I am inclined to agree with Kruse and others that the lactic acid organisms resemble more closely the genus *Streptococcus* than the genus *Bacterium*. Their growth on solid media and their fermentation characters resemble those usually associated with streptococci. The name *Streptococcus lacticus* I is suggested for the largest group (Table II), characterized by the fermentation of dextrose, lactose, maltose, salicin, and mannitol and their inability to attack saccharose, raffinose, and inulin. *Streptococcus lacticus* II may be considered as differing from the former group in its ability to ferment saccharose in addition to dextrose, lactose, maltose, mannitol, and salicin.

SUMMARY.

A well defined group of rod-like and coccoid organisms arranged in pairs and chains has been encountered in sour milk. The group comprises at least three species; the largest number ferment dextrose, lactose, maltose, mannitol, and salicin, and fail to ferment saccharose, raffinose, and inulin. A smaller number ferment saccharose in addition to dextrose, lactose, maltose, mannitol, and salicin. A few fail to attack mannitol. All three types grow luxuriantly at room temperature, coagulate milk, reduce litmus, and produce large amounts of acid in fermented bouillon containing dextrose.

Specific morphological and cultural differences exist between the lactic acid streptococci and those associated with mastitis and those occurring in the udder. The lactic acid organisms outgrow the udder streptococci in the milk-souring process. When both types are implanted in sterile milk the udder type soon disappears.

PREPARATION OF COLLODION SACS FOR USE IN BACTERIOLOGY.

By FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 28, 1920.)

Metchnikoff, Roux, and Taurelli-Salimbeni[1] state that Fick in 1856 was the first to suggest the use of semipermeable collodion membranes in biology. Certainly a general interest in the properties of these membranes from the bacteriological standpoint dates from their own report in 1896 of the intoxication of guinea pigs from the diffusion of cholera toxin through the walls of collodion sacs containing cultures of the vibrio and placed in the peritoneal cavity. Since then a considerable number of articles on collodion sacs have appeared, which it is not within the province of this paper to review. It suffices here to note that the use of collodion sacs has been almost constantly attended with difficulties in manufacture, standardization, and mode of employment which have precluded their widespread use in bacteriology. There is at present no established bacteriological technique involving the intraperitoneal implantation of collodion sacs.

Heretofore most of the studies, whatever their primary object, have come at last to deal mainly with the properties of collodion sacs themselves, especially their permeability to a variety of substances, rather than with their use as an instrument for the furtherance of bacteriological research. The exceptions, however, are noteworthy. In addition to the demonstration of a soluble diffusible toxin of the cholera vibrio, noted above, mention may be made of Vincent's[2] enhancement of the virulence of saprophytes, *B. megaterium* and *B. mesentericus*, by collodion sac passage through rabbits; of Nocard and Roux's[3] demonstration of changes in collodion sac contents suggestive of the multiplication of inoculated material from pleuropneumonia of cattle; and of Nocard's[4] studies on the relation of human to avian tuberculosis by the growth of the human bacilli in sacs in the peritoneum of the chicken. All these papers appeared in 1896 or 1898 from the Pasteur Institute and with a few exceptions represent the total of the contributions made to bacteriology by the intraperitoneal use of collodion sacs.

---

[1] Metchnikoff, E., Roux, E., and Taurelli-Salimbeni, *Ann. Inst. Pasteur*, 1896, x, 257.

[2] Vincent, M. H., *Ann. Inst. Pasteur*, 1898, xii, 785.

[3] Nocard, M., and Roux, E., *Ann. Inst. Pasteur*, 1898, xii, 240.

[4] Nocard, M., *Ann. Inst. Pasteur*, 1898, xii, 561.

Collodion sacs seem to afford a general method for the cultivation of bacteria in pure culture under conditions approaching the invasion of a host; the bacteria are free to yield soluble diffusible products which might affect the animal and to obtain nutritive substances from the animal tissues, yet they are protected against phagocytic attack and, to some extent certainly, from exposure to antibodies. The successful transmission of one human infection after another to animals previously supposed to be immune emphasizes the importance of new methods and new channels of infection, and, in view of the experiments from the Pasteur Institute already mentioned, suggests the further use of collodion sacs for the maintenance of bacterial growth intraperitoneally.

It was with these considerations in mind, and especially with the object of developing another method by which the inciting agent in certain diseases of unknown origin might be sought, that we have tried to obtain a standardized technique for the manufacture and manipulation of collodion sacs. The requirements are that sacs of suitable size and shape may be easily made in large numbers, that they shall be uniformly strong and highly permeable, and that they may be easily sterilized and handled without danger of contamination before and after incubation in the animal body. In the method to be described these problems have been given especial consideration. While methods formerly in use have been freely drawn upon, certain essential modifications have been found necessary, and it appears desirable to report the technique in detail so that it may be followed with exactness.

Following the method of Prudden and McCrae,[5] as modified by Harris,[6] the collodion sacs here described were made on a gelatin capsule foundation which was then dissolved out with hot water. The procedure is as follows:

Preparation of the Sacs.

A small piece of thin walled glass tubing 5 by 0.5 cm., the edges of which have been smoothed in the flame, is heated slightly at one end and pressed against the rounded end of the cap of a large veterinary

[5] McCrae, J., *J. Exp. Med.*, 1900–01, v, 635.

[6] Harris, N. MacL., *Centr. Bakt., 1te Abt., Orig.*, 1902, xxxii, 74.

capsule (4.8 by 1.6 cm.),[7] to which it adheres. With a hot wire a hole is burned through the cap into the glass neck. The open end of the body of the capsule is momentarily dipped just beneath the surface of very hot water, applied to the cap, and thus sealed in with an overlap of about 2 mm. Slight suction with the lips discloses whether the joint is tight. If not, a drop of hot water is touched to the leak. After drying, this framework is ready for the first dip in collodion.

Collodion solutions of two different consistencies are required, one for reinforcement, the other for the permeable membrane. After some experiment Squibb's collodion U. S. P. IX was found satisfactory, though a 4 per cent collodion, as a basis, may be made up from the formula, absolute alcohol 25 parts, ether 75 parts, pyroxylin 4 parts. Squibb's 4 per cent collodion or this solution of pyroxylin is too thin to give a proper membrane with one dip, but higher percentages of pyroxylin do not readily dissolve, and it is found most convenient to obtain the thicker solutions by evaporation. This is accomplished under vacuum with the application of gentle heat (water bath). The collodion boils rapidly, and evaporation is measured by the loss in volume.

The 12 per cent collodion required for the permeable membrane and the 14 to 15 per cent collodion required for reinforcement are obtained by evaporating 4 per cent collodion to one-third or less of its original volume. For evaporating and for dipping, museum jars 20 by 6.5 cm.,[8] with clamped tops and rubber gaskets, are used.

Of the entire surface of the gelatin capsule, the body is to be covered by the permeable membrane of the sac, and the cap by the heavier impermeable wall of collodion which serves to strengthen and support the membrane. This impermeable part is made first by dipping the inverted gelatin capsule (held by wedging the somewhat conical body into the end of a test-tube) neck downward into the 15 per cent collodion to a depth that just covers the joint between body and cap.[9] The capsule is dipped and withdrawn slowly to avoid air bubbles in

[7] Parke, Davis and Company, Detroit, Mich., Empty Capsules No. 12. A capsule of any suitable size may be used.

[8] Whitall Tatum and Company, Philadelphia, Pa.

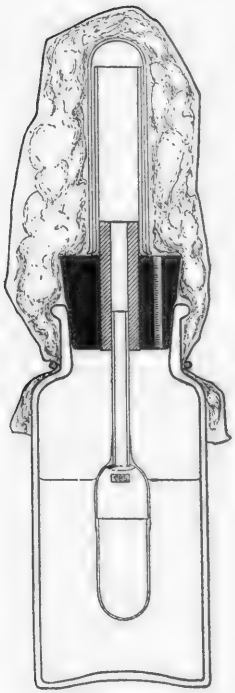
[9] The open end of the glass neck may be covered with a bit of adhesive plaster.

the angle between neck and shoulder and to allow excess collodion to run down the neck. After a few moments the collodion sets and ceases to flow, and the capsule, still held in the test-tube mouth, is inverted and set aside to dry. Before drying is complete the excess collodion is cut away from the glass neck with a knife, leaving a collar 7 mm. high that effectually joins sac and neck together when it dries. This heavy, dry collodion membrane seals and reinforces the joints between body and cap and between cap and glass neck.

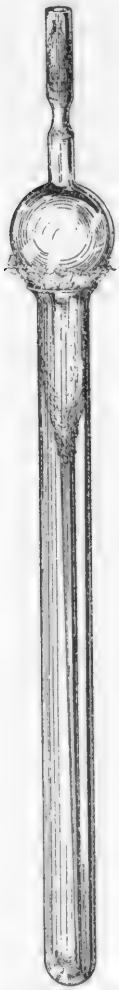
The sacs may be identified by means of small numbered paper labels sealed into the wall. Tiny rectangles of very thin paper are numbered with India ink. These are cut apart, curved slightly on a blotter with the rounded end of a test-tube, dipped with forceps into thin collodion and applied to the dry membrane just below the shoulder, where they adhere and become a permanent part of the wall (Text-fig. 1).

The capsules are now ready for the final dip. They are to be handled, after the sacs are made, in 120 cc. (4 ounce) wide mouth bottles with rubber stoppers. These stoppers have one hole centrally placed for the glass neck, and a second peripherally, which equalizes pressure in the bottle and the sac. On account of its greater elasticity and resistance to autoclaving, a length of stethoscope tubing makes a good core for the central hole in the rubber stopper. The hole is cut with a cork borer slightly smaller than the tubing, which is seized with forceps, pulled through with a projection of 1 cm. on the upper side of the stopper, and cut off flush with the bottom. The use of the projection will be described later.

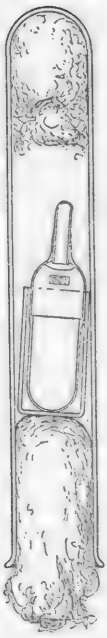
The neck of the reinforced capsule is inserted into the rubber stopper. The capsule is then slowly lowered into 12 per cent collodion in the jar with a rotary to and fro motion which reduces the incidence of air bubbles. It is immersed to the neck in the collodion, so that the joints at neck and body are reinforced. On withdrawal, the coated capsule is suspended above the collodion by means of a slotted card slipped under the stopper and is allowed to drain a definite length of time, as will be described. The membrane dries very little during this draining because of the ether-alcohol tension above the collodion. If a drop hangs from the capsule at the end of the drainage time it is removed by touching it to the surface of the collodion solution. The



TEXT-FIG. 1.



TEXT-FIG. 2.



TEXT-FIG. 3.

TEXT-FIG. 1. Cross-section of a completed collodion sac in its protecting bottle. The various steps in the preparation of the sac are explained in the text.

TEXT-FIG. 2. A Pasteur pipette prepared for dry sterilization.

TEXT-FIG. 3. A sac in its cup and test-tube, after the neck has been sealed off in a small blast flame.

capsule is rapidly withdrawn from the jar and inverted to dry in the air for 1 to 2 minutes. It is then lowered into 95 per cent alcohol in a 4 ounce wide mouth bottle, where the collodion membrane finally sets.

The capsule with its collodion coating may remain in the alcohol indefinitely, but a few minutes are sufficient to extract the remaining ether and saturate the membrane with 95 per cent alcohol. As the viscosity of the collodion and the drainage determine the thickness of the sac, so the drying and the alcohol treatment determine its permeability.

After treatment in 95 per cent alcohol the capsule is plunged into cold water until the alcohol is removed and the gelatin capsule framework softens, a matter of an hour or less. The gelatin is then thoroughly washed out with a stream of hot water. For this purpose we have used a board with holes to accommodate twelve stoppers, resting on a basin of hot water. From the faucet, distributing tubes, ending in glass capillaries which are just long enough to reach into the sacs but not to pierce their bottoms, carry in the hot water and so wash the dissolved gelatin out through the neck. An hour or so of washing leaves the completed sac, attached to its glass neck by the collar of heavy collodion. It is then emptied with a Pasteur pipette and tested for strength and imperfections. Each sac must withstand an internal pressure of at least 25 cm. of mercury (5 pounds per square inch). The empty sac is immersed in distilled water in a 4 ounce bottle, and the second hole in the stopper is connected by a stub of glass tubing with a vacuum pump and gauge. The air in the bottle is exhausted 25 cm. A stream of air bubbles from the surface of the sac reveals any leak. If bubbles in the collodion were carefully avoided a leak is rare. Imperfect sacs are not worth repairing and are discarded.

The sac is then filled to the neck with distilled water and is ready for the autoclave. The expansion of water on autoclaving is sometimes sufficient to cause an overflow, and consequent loss of water on cooling. The projection of the stethoscope tubing above the stopper is therefore fitted with a short length of glass tubing open at the top. This forms an expansion chamber, from which the water drains back into the sac on cooling. To prevent contamination the glass tube is capped by a short, loosely fitting, inverted test-tube.

The top of the bottle is swathed in raw cotton and covered with paper, tied on. In this way contamination of either the inside or outside of the sac is avoided, and the sac is protected indefinitely for future use. Ten or twelve sacs are thus prepared at one time and then autoclaved at 15 pounds pressure for 30 minutes (Text-fig. 1).

Mode of Use.

Before discussing permeability, which is the vital factor in the collodion sac technique, the mode of inoculation and peritoneal implantation may be described briefly.

The most convenient instrument for filling and emptying the sacs and preserving their contents is a Pasteur pipette. Those which we use are made in quantities in the laboratory from 24 cm. lengths of rather heavy glass tubing pulled out in the middle and sealed off to make two pipettes. A bulb about 2 cm. in diameter is then blown just above the shoulder. The requisites are very hot glass and gentle pressure. The tip is cut just to reach the bottom of a Noguchi culture tube (20 by 1.5 cm.), and a wrapping of cotton below the bulb acts as a stopper for the tube, in which the pipette is dry sterilized (Text-fig. 2). With these pipettes the sacs are filled, and after incubation the sac contents are similarly withdrawn and transferred to the test-tube which has protected the pipette from contamination. Then the pipette with its cotton collar serves as a plug for the tube and is used for subsequent withdrawal of fluid for examination.

Inoculation of small quantities of material into media in the sacs is accomplished with a platinum loop or a small sterile Wright pipette plugged with cotton. Such a pipette, flamed, and bent into a V saves the use of a Pasteur pipette in emptying out the distilled water from the sterilized sac preparatory to use. The bottle is inverted, the small arm of the pipette inserted upward through the neck, and gentle breath pressure expels the distilled water without danger of air contamination.

After being filled to the neck with a suitable medium and inoculated with bacteria or suspected material, the sac is withdrawn from the protecting bottle and the neck sealed off smoothly in a small blast flame. During this process and subsequently, contamination of the outside of the sac is avoided by carrying it in a short glass cup with

a flat bottom so that it will stand upright. Those used in the present work were made from 6 dram homeopathic vials cut off with a hot wire and a plunge into cold water. They are wider than the sacs and about 3 cm. long, so that the shoulder and neck project. The cups are sterilized by dry heat in wide test-tubes. When the neck of the sac is to be sealed off, one hand holds a cup containing the sac, while the other hand manipulates the rubber stopper. The cup, with its sac, is reinserted into the inverted test-tube until the neck has cooled (Text-fig. 3). The neck is then painted with a layer of collodion, which fuses with the collodion collar and so prevents the possibility of leakage between collar and neck. After a few minutes drying the sac is ready for intraperitoneal insertion. This is done aseptically under full ether anesthesia through a short incision in the abdominal wall above the umbilicus. The surgical technique need not be described, except to say that the incision should be sewed up firmly in layers. Metal skin clips save time. A dressing of cotton and collodion is sufficient. No bandage is required.

As many as eight such sacs have been tolerated by an adult rabbit without any apparent discomfort. The tissue reactions which often occur around the sacs will be discussed later. After an appropriate incubation period the rabbit is killed by an occipital blow and the sacs are recovered through a wide abdominal incision. They are again dropped into the glass cups, opened at the shoulder with a hot wire loop, and their contents transferred to a Noguchi tube by means of its Pasteur pipette.

General Characteristics of the Sacs.

Sacs made as just described have a capacity before autoclaving of from 6.5 to 7 cc. The upper third is a tough, impermeable wall which supports the glass neck and the permeable bottom. The lower two-thirds, with a surface area of about 14 sq. cm., is a thinner, moist, elastic membrane of high permeability. It is heavy enough, however, to stand immersion in water without collapsing and to withstand an internal pressure of 5 pounds per square inch. Such sacs rarely break during incubation. The walls of the sac are colorless and transparent.

Autoclaving at 15 pounds pressure for 30 minutes causes a uniform shrinkage to a capacity of 4.7 to 4.2 cc., corresponding to a diminution in surface area of approximately 25 to 30 per cent. Permeability is somewhat diminished but remains high. The shape and transparency of the sac are unaltered. The sacs may be kept sterile in distilled water in their bottles for months without appreciable loss in permeability, but the walls tend to become more brittle and inelastic.

Permeability.

The permeability of the membranes has been tested in a number of ways. The quantitative test by which variations in materials and methods of manufacture were compared consisted in dialyzing a 2 M solution of sodium chloride within the sac against 20 or 25 volumes of distilled water and titrating a sample of the dialysate from time to time with 0.02 M silver nitrate solution, sodium chromate being used as indicator. The quantitative relations were such that when equilibrium was reached 1 cc. of the dialysate would precipitate 5 cc. (or 4 cc.) of the silver solution as silver chloride.

In 1915 Brown[10] contributed a careful study of collodion membranes and showed in particular that the permeability of membranes could be accurately controlled by complete drying, followed by immersion in ethyl alcohol of a definite dilution. Alcohol dilutions below 30 per cent confer practically no permeability on an air-dried membrane. 95 per cent alcohol produces a high degree of permeability. In preliminary experiments Brown's results were fully confirmed, and the value of his discovery was appreciated. His membranes, however, were prepared for chemical dialysis and did not require sterilization. On trial it was found that air-dried membranes, rendered permeable by alcohol treatment, lost their permeability again upon sterilization by heat. On the other hand, sacs which were not allowed to dry before immersion in alcohol lost little in permeability by sterilization in the Arnold sterilizer or the autoclave. Since highly permeable sacs were desired, 95 per cent alcohol was used as a routine.

Viscosity of the Collodion.—The percentage of pyroxylin in the collodion determines its viscosity, and viscosity in turn determines

[10] Brown, W., *Biochem. J.*, 1915, ix, 591.

the thickness of the collodion coating that will adhere to the dipped capsule. On account of rapid evaporation of the solvents the solution must be tested frequently and maintained at approximately the proper density. Commercial collodions vary considerably in density, and it is not a safe rule to evaporate a so called 4 per cent or U. S. P. collodion to one-third of its volume to obtain a 12 per cent solution.

The percentage of pyroxylin in the solution may be measured directly by weighing a specimen before and after evaporation to dryness. But this is not a convenient method, except as a check and to standardize a simple viscosimeter by which the thick collodions may be readily tested and compared.

The viscosimeter which we use consists merely of a 15 cm. length of Pyrex glass tubing, with square-cut ends, the internal diameter of which happens to be 0.365 cm. Near the middle of this tube file marks measure a distance of 5 cm. The tube is dipped into the collodion to be tested, a column is drawn up by suction well beyond the upper file mark, and the tube is withdrawn. As the column is then released in the vertical tube a stop-watch is used to time the meniscus over the measured distance of 5 cm. 12 per cent collodion at 20°C. requires 15± seconds to flow past the marks on the viscosimeter used.

Having standardized such a simple instrument it is easy to maintain thick collodion at the proper density by frequent tests and the addition of ether and absolute alcohol 3:1 to compensate for evaporation.

An even simpler method of determining viscosity, and one which is probably sufficiently accurate for the purpose, is to time the flow of collodion from a dipped capsule until the stream breaks and is succeeded by a series of drops. This time interval increases rapidly with the density of the collodion. In one test, for example, it increased from 12 seconds with a 10 per cent collodion to 1 minute, 15 seconds with the same collodion evaporated to approximately 12.7 per cent. The stream should flow about 1 minute before breaking.

The heavier collodion used for the neck and shoulder of the sac need not be standardized. We use it so thick that it will just flow smoothly, with no tendency to "jell." Collodion that has been used for permeable membranes until it has lost its clear transparency will serve the purpose.

Determination of the Draining Time.—The length of time that drainage is allowed to proceed influences the thickness of the collodion

coating and the evenness of its distribution over the surface of the capsule. Provided the first few drops are permitted to fall after the stream of collodion has broken, little is gained by prolonging the drainage. As will be shown later, slight variations in thickness are of minor importance, and sufficient uniformity is obtained by allowing a definite number of drops to form and fall.

Experiment 1.—From five sacs, after dipping, an unbroken stream of collodion flowed for an average of 1 minute, 16 seconds. Successive drops then fell after 1 minute, 26 seconds; 1 minute, 52 seconds; 2 minutes, 32 seconds; and 3 minutes, 29 seconds on the average. After 2 drops had fallen, 40 seconds were required to reduce the thickness of the membrane by the amount of 1 drop of collodion, and 57 seconds were required for the following drop to form.

It is hardly profitable to prolong the drainage time beyond the separation of the first few drops.

Effect of the Drying Time on Permeability.—

Experiment 2.—Five collodion sacs were made on No. 12 gelatin capsules by dipping once in 12 per cent collodion, draining 2 minutes, and drying for various intervals, as shown in Table I, before immersion in 95 per cent alcohol for 1 hour.

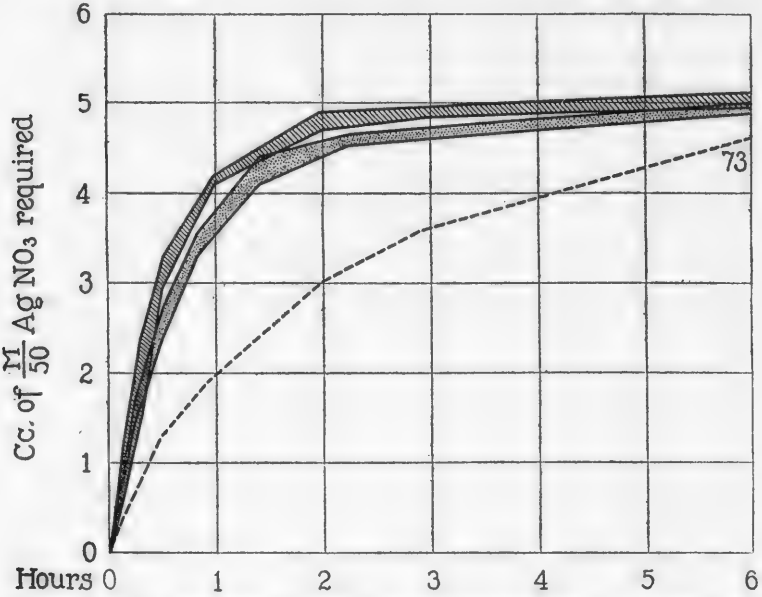
TABLE I.

Sac No.	Drying time.	Sterilization.	Capacity.	
			Before sterilization.	After sterilization.
			cc.	*cc.*
81	0	Arnold sterilizer, 3 hrs.	6.4	4.6
76	15 sec.	Autoclave, 30 min.	6.2	4.8
82	30 "	Arnold sterilizer, 3 hrs.	6.4	4.7
80	1 min.	Autoclave, 30 min.	6.1	4.6
73	1 hr.*	Arnold sterilizer, 3 hrs.	7.3	6.2

* Completely dry in 1 hour.

The permeability of the sacs to sodium chloride was determined both before and after sterilization and is shown in Text-fig. 4. Sodium chloride diffused much more rapidly through the sacs which were not completely air-dried before the alcohol treatment. Sterilization, whether in the Arnold sterilizer at 100°C. or in the autoclave at 15 pounds pressure, did not greatly impair the permeability, notwithstanding the shrinkage which accompanied the process. The air-dried sac,

No. 73, on the other hand, shrank less during sterilization, but its permeability was so far lost in the Arnold sterilizer that no measurable amount of sodium chloride had passed through it in 6 hours, and only 1.30 units (on a scale of 5) had passed in 48 hours.

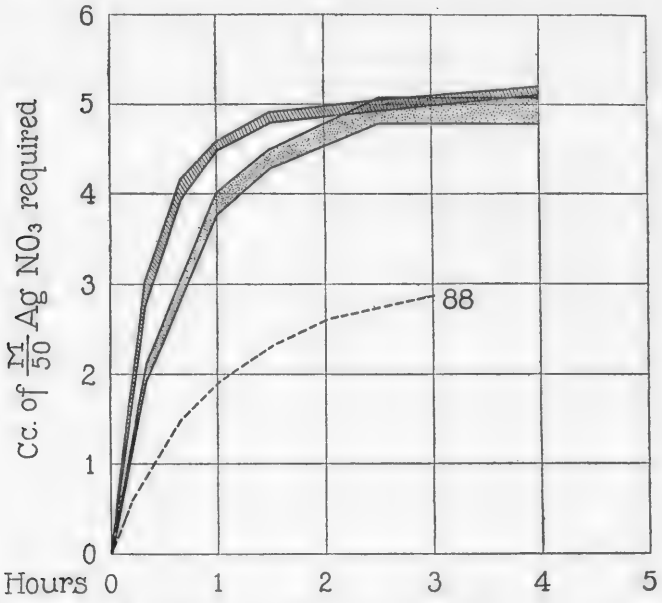


TEXT-FIG. 4. Experiment 2. The cross-hatched area covers variations in permeability of Sacs 81, 76, 82, and 80 (Table I) before sterilization. The stippled area covers variations in permeability of these sacs after sterilization. The broken line is the permeability curve of Sac 73, dried before the alcohol treatment. Sac 73, after sterilization, passed no measurable amount of sodium chloride in 6 hours.

Experiment 3.—In a similar experiment four sacs were dipped once, drained 3 minutes, dried 1, 2, 3, and 4 minutes respectively, and treated with 95 per cent alcohol over night. Their capacities before sterilization were 6.3, 6.1, 6.0, and 6.5 cc., and they shrank to 4.9, 4.7, 4.7, and 5.3 cc. in the autoclave. Their permeability before and after autoclaving is shown in Text-fig. 5.

From these two experiments it is evident that variations in the drying time have little influence on permeability, provided the membrane is still moist when immersed in alcohol. Sacs dried for 30

seconds to 1 minute are the most satisfactory. If plunged immediately into alcohol after drainage they tend to wrinkle and to show a smoky bluish opacity, and they are not so tough and strong as the partly air-dried sacs.



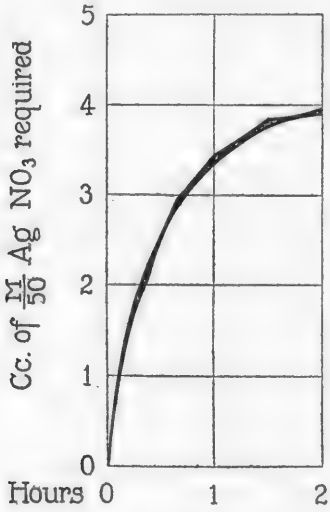
TEXT-FIG. 5. Experiment 3. The cross-hatched area covers variations in permeability of four sacs before sterilization. The stippled area covers variations in permeability of the same sacs after sterilization. The broken line is the permeability curve of Sac 88, not treated with alcohol before sterilization.

Importance of the Alcohol Treatment.—Other experiments indicate that treatment in 95 per cent alcohol for as short a time as 1 minute is sufficient to insure sacs of high permeability. It is only necessary that the alcohol should replace the ether-alcohol solvent of the collodion before the membrane is set by immersion in water. The importance of the alcohol treatment is illustrated in Text-fig. 5 by the permeability curve of Sac 88, which was drained 1.5 minutes, dried 30 seconds, and then plunged into water without the preliminary

immersion in alcohol. Although this sac was made of the same collodion as the others and shrank only from 6.9 cc. to 6.5 cc. during sterilization, its permeability to sodium chloride was much less than that of alcohol-treated sacs.

Shrinkage of the Sacs during Sterilization.—The hot water used to wash out the gelatin framework of the sacs causes an initial loss in capacity from 7+ cc. to about 6.6 cc. Sterilization by heat further shrinks the membrane to an average capacity of 4.5 cc. The loss in capacity and permeability due to heating is absolute and does not continue once the limit is reached.

Experiment 4. Effect of Re-Autoclaving.—Four sacs, dipped once in 12 per cent collodion, drained 1.5 minutes, dried 30 seconds, and immersed in 95 per cent alcohol for 1 hour, were autoclaved at 15 pounds pressure for 30 minutes. Two of the sacs were then re-autoclaved under similar conditions. The capacity of the first two sacs was 4.5 and 5.1 cc. respectively, of the second two 4.4 and 5.05 cc. All four sacs had a practically identical permeability as shown by Text-fig. 6. The curve incidentally illustrates the uniformity of collodion sacs made as described above.



TEXT-FIG. 6. Experiment 4. The solid black area covers variations in permeability of four sacs, two of which were autoclaved once, the other two autoclaved twice.

The relation between the diminution in capacity and in surface area of the sacs on sterilization is shown in the following experiment.

Experiment 5.—Eight sacs made in an identical manner from the same collodion solution were divided into two groups of four each. After volume measurements had been taken, four of the sacs were autoclaved and their capacities again determined. All the sacs were then cut open so that they could be flattened out, shadowgraphs were made on photographic paper, and the areas measured with a planimeter. The average measurements are shown in Table II, in which are included similar figures for two sacs that were completely dried before the alcohol treatment.

TABLE II.

Condition and No. of sacs.	Volume.				Surface.			
	Before autoclaving.		After autoclaving.		Before autoclaving.		After autoclaving.	
	Body.	Total.	Body.	Total.	Body.	Total.	Body.	Total.
	cc.	*cc.*	*cc.*	*cc.*	*sq. cm.*	*sq. cm.*	*sq. cm.*	*sq. cm.*
Two dried sacs.			4.9	6.95			14.3	21.9
Four undried sacs; not autoclaved.	4.5	6.72			14.0	21.6		
Four undried sacs; autoclaved.	4.45	6.67	2.72	4.5			9.6	15.8
Capacity loss.			39 per cent.	33 per cent.			31 per cent.	27 per cent.

It is seen from Table II that sterilization of the permeable sacs caused a loss of 33 per cent in capacity, corresponding to a surface shrinkage of 27 per cent. The losses were not proportionately distributed between the impermeable cap and the permeable body, which lost 39 per cent in capacity and 31 per cent in surface area.

This shrinkage on heating is due largely to the alcohol treatment of undried sacs. Dried sacs, even though treated subsequently with alcohol, shrink but little in the alcohol, as Brown[10] has shown, or in the autoclave (Table II). Undried sacs, not treated with alcohol, also shrink but little on sterilization. The part played by shrinkage in decreasing permeability has probably been overestimated. That the shrinkage itself is of minor importance is shown by a comparison

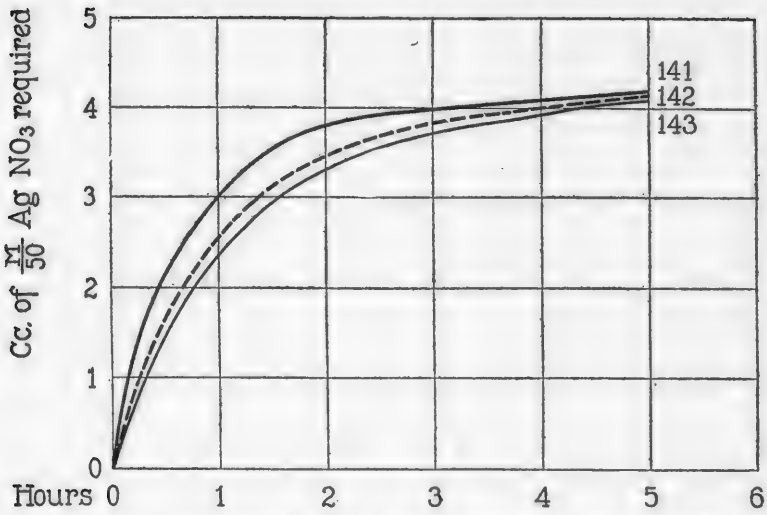
of the permeability of alcohol-treated undried sacs with that of sacs prepared by other methods.

In this connection it may be pointed out that titrations with sodium chloride do not give complete information with regard to the permeability of the sac walls. The sodium chloride molecules and their constituent atoms are of relatively small size, and the rate at which they diffuse through the permeable membranes suggests that the pores of the membrane must be several times the size of the molecules to permit such rapid passage. The volume shrinkage on sterilization is approximately 33 per cent, which corresponds to a diminution in permeable surface area of 31 per cent. Assuming that the pores diminish correspondingly, it is conceivable that while small molecules might still pass through with ease, other molecules which more nearly approached the unshrunken pores in size might now be entirely held back. So far, however, among the substances tested none has been found which passes through the unheated, but not through the heated membranes.

Relation of Thickness to Permeability.—Another factor emphasized in permeability experiments with collodion membranes is thickness. In the present method, within limits, this factor also is found to be secondary in importance to the alcohol treatment. The viscosity of the collodion solution and the drainage and drying time are the variables in determining thickness and have been experimentally controlled (Experiments 1, 2, and 3). A 12 per cent solution forms a sac of sufficient strength for intraperitoneal incubation. Thicker sacs, however, may be used without great loss in permeability.

Experiment 6.—Three collodion sacs were made on a gelatin capsule framework. No. 141 was dipped once in 12 per cent collodion, No. 142 twice, and No. 143 three times. The sacs were drained 2 minutes and dried 45 seconds between each dip. All were immersed in 95 per cent alcohol for 1 hour, washed, and autoclaved. Sac 141 was thin and transparent. Sac 142 was of medium thickness, with a slightly smoky opacity. Sac 143 was very thick and tough, with a smoky opacity. Capacities before autoclaving, 6.8, 6.1, and 6.4 cc. respectively; after autoclaving, 4.7, 4.0, and 4.2 cc. Such sacs in their thinnest area, just below the joint, were found to measure 0.09, 0.31, and 0.52 mm. in thickness after sterilization.

The results of permeability tests with 4 cc. of 2 M sodium chloride against 96 cc. of distilled water are shown in Text-fig. 7.



TEXT-FIG. 7. Experiment 6. The permeability curves, after sterilization, of three sacs of widely different thicknesses. Sac 141 dipped once in 12 per cent collodion, Sac 142 dipped twice, and Sac 143 dipped three times. In this experiment equilibrium in dialysis is represented by 4 cc. of silver nitrate. The error in the final readings is due to the removal of successive portions of dialysate for test. This loss in volume was not compensated during the experiment.

The difference between the sacs is less than would be expected from the comparative thickness of their walls and indicates that slight variations in thickness would be of no practical significance.

Qualitative Tests of Permeability with Other Substances.—In addition to the titrations of permeability with sodium chloride a number of qualitative tests with other substances have been made. These tests were incidental to other experiments and are therefore incomplete, but the results are useful in indicating the relative permeability of the sacs.

1. Various inorganic salts passed through the sacs with an ease approaching that of sodium chloride. With such substances the endosmotic pressure of a concentrated solution is so quickly lowered by passage through the sac that the level of liquid within the sac is hardly raised significantly before equilibrium is established.

2. Oxygen in solution rapidly diffuses through the sac wall and restores the color to methylene blue reduced by the action of dextrose broth. This fact probably explains the consistent failure of representative strict anaerobes to grow in permeable sacs intraperitoneally implanted. Haggard and Henderson[11] estimate the oxygen tension of the peritoneal cavity at about 45 mm. So far no method has been devised by which this handicap may be overcome.

3. During intraperitoneal incubation the hydrogen ion concentration of sac contents tends to come to an equilibrium with that of the peritoneal fluid. Ascitic fluid, dilute rabbit serum, and broth, for example, whatever their initial reaction, and $\frac{M}{15}$ phosphate mixtures, in proportions to give various pH concentrations, have all been reduced to a pH of 7.4 to 7.5. *In vitro* it is found that the primary and secondary phosphates diffuse through the walls with equal facility. The dialysate comes almost immediately to the pH concentration of the phosphate mixture used.

4. Among the simpler organic compounds, dextrose alone has been tested. It soon shows its presence on the opposite side of the membrane, but diffuses more slowly than do inorganic salts and so gives opportunity for a considerable endosmosis before equilibrium is established.

5. Nutrient materials of meat infusion broth pass through the sacs with sufficient rapidity to promote a luxuriant growth of bacteria (*B. typhosus*, *B. pyocyaneus*) inoculated into distilled water. The straw-colored pigments of the broth are likewise diffusible. Nutrient materials are obtainable from body fluids in a similar manner. Heavy growths of *B. pyocyaneus*, *B. typhosus*, and Type I pneumococcus were obtained by incubating inoculated sacs of distilled water over night in a rabbit. *B. pfeifferi*, lacking hemoglobin, did not grow under similar conditions.

6. The diffusible products of bacterial metabolism have not been determined by analysis. That the sacs are permeable to these products is shown by the tissue reactions which occur around actively growing cultures. The sacs themselves are practically inert, and it is common to find uninoculated control sacs lying free among the intestines without any observable irritation of the surrounding tissues. Sacs which contain living bacteria, on the other hand, are usually the center of an active proliferative process. They are found wrapped in folds of thickened and injected omentum or among intestinal adhesions, sections of which show fibrin deposits, accumulations of leucocytes, rarely to the extent of pus formation, localized hemorrhages, infiltration with fibroblasts, and newly formed capillaries; in short, the various elements and stages of a degenerative and regenerative tissue reaction.

7. No evidence has been observed that hemoglobin or other unsplit proteins will pass through the sacs. In two experiments in which highly potent antimeningococcic serum was surrounded by a suspension of meningococci no agglutination occurred.

---

[11] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxviii, 71.

In summary, it may be said that the sacs as described are permeable to gases in solution, to inorganic salts, to dextrose, to certain protein-split products which are nutritive to bacteria, and to certain toxic products of bacterial metabolism, but they hold back antibodies, unsplit proteins, and formed elements such as bacteria and body cells.

SUMMARY.

A standardized method is described in detail by which collodion sacs suitable for intraperitoneal incubation and for other bacteriological experiments may be produced in large numbers, sterilized, and handled with convenience and the minimum danger of contamination. Various factors influencing permeability have been subjected to experiment. Like Brown, we found that immersion in alcohol is the most important factor, but the high permeability conferred by alcohol treatment is lost during heat sterilization if the membrane was previously allowed to dry. Quantitative experiments on the dialysis of sodium chloride, and simple tests with other substances indicate the general character of the membranes and their probable field of usefulness in bacteriology.

A MECHANICAL MEASURING INSTRUMENT FOR STERILE LIQUIDS.

By FREDERICK L. GATES, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 28, 1920.)

Part of the strain and tedium of immunological research is connected with the distribution of identical portions of a given liquid among a large number of tubes. Ordinarily this task is performed by means of a graduated pipette, in which the movement of the liquid is controlled by the ball of the finger and regulated by watching the meniscus as it passes the graduation marks. The process is time-consuming, and the precise control necessary to stop the meniscus exactly on the mark is a tax on eye and hand.

The instrument here described[1] was first devised as a substitute for the graduated pipette in immunological tests. Since it mechanically measures any amount of liquid within its capacity in a sterile manner it may find application also in serum and vaccine laboratories, in media preparation rooms, and wherever aliquot portions of a liquid are to be measured out.

As with the graduated pipette, the principle involved is the displacement of the required amount of liquid by a similar amount of air at atmospheric pressure. The air is measured by a graduated syringe, which may be set to deliver any amount within its capacity. Before coming in contact with the liquid, the air is twice filtered through raw cotton plugs. Provided the container is sterile all danger of contamination is eliminated.

Description of the Instrument.

The syringe *F*, of any required capacity, is screwed into one outlet of a three-way stop-cock *H*, which also supports a rigid hollow sleeve

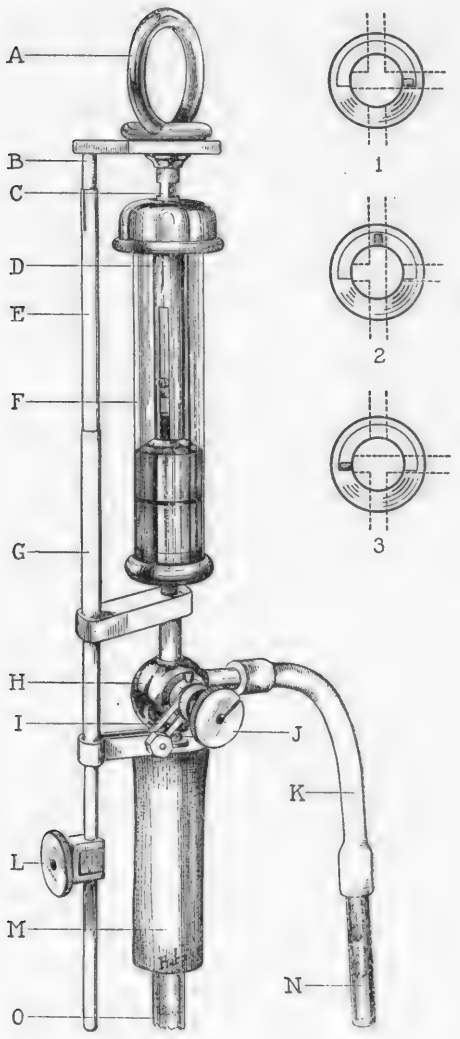
[1] The instrument may be obtained from the Central Scientific Company, 460 East Ohio Street, Chicago, Ill.

G and forms the frame of the instrument. This sleeve *G* guides an inner sliding sleeve *E* which operates the key of the stop-cock through the slotted arm *I* and is itself operated by friction contact with the sliding rod *B*. This rod engages the plunger rod *C* of the syringe, and by means of the adjustable lock-nut *L* limits its travel. The finger, slipped through the ring *A*, raises and lowers the plunger rod and operates the stop-cock mechanism. The plunger is surmounted by a slotted sleeve *D* in which the plunger rod fits loosely and operates the plunger only toward the end of the stroke in either direction. The object of these sliding mechanisms is to turn the key of the stop-cock before the plunger moves.

In Text-fig. 4 the instrument is pictured at the end of the downstroke. The stop-cock is open in the direction indicated in Text-fig. 2. A complete up- and downstroke may be followed in detail. As the finger, slipped through *A*, raises the plunger rod *C*, the rod *B* is raised also, carrying with it, by friction contact, the sleeve *E*. This sleeve in turn operates the key of the stop-cock through the slotted arm *I* so that during the travel of *E* the key turns from position 2 (Text-fig. 2) to position 1 (Text-fig. 1). During this first part of the upstroke the plunger does not move, since the plunger rod slides freely in the loose sleeve *D*. When the stop on *C* reaches the end of the slot in *D*, the sleeve *E* also reaches the frame *G*, and the key of the stop-cock reaches position 1. The stop-cock is now open for the admission of air to the syringe barrel through the side outlet of the cock and the short rubber tubing *K* and cotton filter *N*.

As the finger continues to raise the plunger rod the plunger is now raised also by the engagement of the stop on the rod in the end of the slot in *D*. The rod *B* slides through sleeve *E* until its travel is stopped by contact of the lock-nut *L* with sleeve *E*, which is already in contact with *G*. The plunger has now risen in the barrel a definite distance, determined by the position of the lock-nut *L* on the rod *B*, and a measured quantity of air at atmospheric pressure is contained within the syringe.

On the downstroke the first movement is again that of *A*, *B*, *E*, and *I*, operating to turn the key of the stop-cock back to position 2. This accomplished, the plunger rod *C* engages the sleeve *D* at the bottom of the slot and drives the plunger down. The air in the syringe



TEXT-FIGS. 1, 2, and 3. Various positions of the stop-cock key. (1) Position for the admission of air to the syringe; (2) for the expulsion of air into the liquid container; (3) for filling the container by suction.

TEXT-FIG. 4. The complete instrument. The lettering is explained in the text.

barrel is expelled into the container *O* attached to the heavy rubber tubing *M*, and displaces a corresponding quantity of liquid which is thus delivered by the apparatus.

The instrument may be set to deliver any volume of air, and consequently any volume of liquid, within the capacity of the syringe. To set it for a given volume the lock-nut *L* is loosened and the plunger raised exactly to the desired graduation mark on the syringe barrel. Then the lock-nut is set snugly against the sleeve *E*, in contact with *G*, and tightened.

Any sort of container with an inlet for the measured air and an outlet for the liquid may be used. The only requirement is that the system shall be air-tight except at the intake and outlet. The instrument may be attached to it directly or through a length of tubing. The elasticity or rigidity of the walls is of no consequence, since the system returns to atmospheric pressure after each delivery.

In immunological tests in which relatively small amounts of liquids are handled, a 5 or 10 cc. pipette may be used. The instrument is operated with one hand, and the pipette held and directed with the other. Larger amounts of liquid may be contained in flasks or bottles with a tightly fitting two-hole rubber stopper and a delivery tube reaching to the bottom. In such instances the instrument may be clamped in a frame and the rod *B* operated by a pedal, leaving both hands free.

When a pipette is used it is refilled as follows: The plunger is raised to the top of the stroke, and the split thumb-nut *J* partly unscrewed. It first releases the slotted arm *I*, and then, by friction, it turns the key of the stop-cock from position 1 to position 3 (Text-fig. 3). The stop-cock is now open from the side outlet to the pipette, which is filled by suction on *N*. The thumb-nut is again tightened, returning the key to position 1 and locking the arm *I* in place.

In order to minimize errors in measurement, attention should be given to several precautions in the use of the apparatus. (1) For exact measurements the syringe must be accurately calibrated and the lock-nut carefully set to bring the plunger exactly to the mark. (2) The system must be air-tight. Since the instrument itself need not be sterilized, the stop-cock and plunger may be lubricated with a heavy oil or grease. The addition of a little beeswax to the plunger

lubricant keeps the plunger in position at the top of the stroke until it is forced down by contact of the plunger rod. (3) An error is introduced by a difference in level between the surface of the liquid in the container and the point of outlet. Usually this error is negligible. A difference in level of 10 cm. causes an error of about 1 per cent. (4) Care must be taken that the air is not expelled too rapidly into the container so as to force the liquid out under pressure, lest more than the required amount be delivered by its own momentum. This is recognizable by the subsequent retreat of the liquid from the delivery tip, as equilibrium is reestablished. A complete stroke should be up, and then down, for each delivery. A small delivery tip lessens the error due to the adherence of the last drop of liquid. (5) When air and liquid are at different temperatures an error is introduced by expansion or contraction of the measured air on contact with the liquid. For accuracy, therefore, the air and liquid should be at the same temperature. Practically, when the instrument is handled with reasonable care, these sources of error are not encountered.

The facility with which sterile solutions may be measured out without contamination should be emphasized. The liquid does not pass through the instrument, and nothing comes in contact with it but twice filtered air.

SUMMARY.

An instrument is described by which aliquot portions of a liquid may be mechanically measured and delivered. It was devised especially for use in immunological experiments, to take the place of a graduated pipette in setting up serum tests. The instrument may be set for any quantity within its capacity and measures sterile liquids without danger of contamination. It may therefore find a wider application in other procedures requiring sterile measurements of small amounts of liquid.

FACTORS INFLUENCING ANAEROBIOSIS, WITH SPECIAL REFERENCE TO THE USE OF FRESH TISSUE.

By FREDERICK L. GATES, M.D., and PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 2.

(Received for publication, June 24, 1920.)

Anaerobic cultivation of bacteria has in recent years developed into an important field of bacteriological research. Anaerobic methods have been extensively applied, for example, to the study of the bacterial flora of war wounds and to the investigation of diseases of unknown origin.

In these investigations the cultivation of anaerobes in tubes containing bits of fresh tissue has been extensively employed. Although study of some of the principles involved has led to improvements in method, a method for the quantitative examination of the factors involved is lacking and the establishment of practical rules and indications to meet the requirements of the more fastidious of this group of microorganisms is still to be attained. We shall attempt to show the influence of certain elements in promoting or in hindering the development of strict anaerobic conditions in culture tubes and so to indicate more exactly the value of these factors in anaerobic cultivation.

Aside from the use of mechanical methods for removing oxygen from ordinary media, much attention has been devoted in recent years to the cultivation of anaerobes in a fluid or semisolid medium containing a fragment of fresh sterile tissue, usually kidney, and overlaid with a layer of paraffin oil. The value of the tissue fragment was pointed out by Theobald Smith[1] in 1899, and rediscovered by Tarozzi[2] and Wrzosek[3] in 1905. The use of paraffin oil is credited to Legros.[4]

[1] Smith, T., *J. Boston Soc. Med. Sc.*, 1898–99, iii, 340.

[2] Tarozzi, G., *Centr. Bakt., Ite Abt., Orig.*, 1905, xxxviii, 619.

[3] Wrzosek, A., *Wien. klin. Woch.*, 1905, xviii, 1268.

[4] Legros, G., Recherches bactériologiques sur les gangrènes gazeuses aiguës, Paris, 1902; *Compt. rend. Soc. biol.*, 1902, liv, 1337.

In 1911 Noguchi,[5] using tall columns of serum water overlaid with paraffin oil in narrow tubes and containing a fragment of fresh tissue, was able to grow *Spirochæta pallida* under strictly anaerobic conditions. He did not rely on the kidney tissue or the paraffin oil for the production of anaerobiosis, but for the first time employed with these a combination of hydrogen gas, vacuum, and pyrogallic acid in an anaerobic jar.

Later, in the cultivation of the globoid bodies of poliomyelitis[6] it was found that the mechanical precautions could sometimes be omitted.

Following these reports the tissue-serum water or ascitic fluid tubes overlaid with paraffin oil came to be widely used as an anaerobic method and some investigators, seeking strictly anaerobic conditions, have disregarded the elaborate precautions which Noguchi employed. But the successful use of the tissue method alone, which appears to be simple, has been found to require considerable patience and experience, and the method has often suffered for lack of quantitative standardization and through misunderstanding of underlying principles.

A number of variables are involved. The first requisite for a study of these variables is a delicate and precise reversible indicator for the presence or absence of free oxygen in solution. The indicator should react in the presence of culture media, so that it may be added directly to the materials to be tested, and should not interfere with the reducing activity of other components of the medium or arrest the growth of test organisms. Methylene blue fulfills these requirements in a satisfactory manner.

Theobald Smith[7] in 1896 reported the reduction of methylene blue and other indicators in the closed area of fermentation tubes by sterile peptone broth. The presence of muscle or grape sugar and the application of heat increased the speed of the reaction. Peptone and dextrose water were inert. Spina[8] had already noted the reduction of methylene blue by nutrient gelatin, but not by agar.

---

[5] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

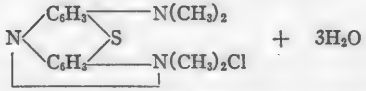
[6] Flexner, S., and Noguchi, H., *J. Exp. Med.*, 1913, xviii, 461.

[7] Smith, T., *Centr. Bakt., Ite Abt.*, 1896, xix, 181.

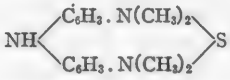
[8] Spina, A., *Centr. Bakt.*, 1887, ii, 71.

Methylene Blue as Indicator.

By the action of weak deoxidizing agents methylene blue (tetramethylthionine chloride)



is converted readily to colorless leucomethylene blue, or α-(p)-tetramethyldiaminothiodiphenylamine



The reaction is reversible, and the conversions proceed rapidly at incubator temperature in the presence of free oxygen or under the attack of the deoxidizing agent after the free oxygen has been consumed.

Experiments were made to determine the sensitiveness of the indicator and the conditions under which it reacts. Ordinarily 0.1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of 2 per cent dextrose peptone broth in a test-tube is placed in the anaerobic jar and the removal of free oxygen is indicated by the gradual decolorization of the dye. Since methylene blue is not decolorized under similar conditions in a medium of distilled water, we sought the ingredient in the dextrose broth which promotes the decolorization.

Relation of the pH of the Medium to Its Reducing Activity.—Beef infusion, 2 per cent peptone solution, and 2 per cent dextrose, with methylene blue, when boiled in a water bath to remove the air were not decolorized. Nor were mixtures of any two, or of all three of the ingredients. But so far in this experiment the hydrogen ion concentration of these mixtures had not been considered. The foregoing materials were retested in a solution of 0.01 M sodium hydroxide. Decolorization was immediate on heating to drive off the air, but the dye was resolved into simpler elements than the leuco form, thus destroying its value as an indicator.

2 per cent dextrose solution, colored with methylene blue, was mixed with an equal volume of buffer phosphate mixtures in $\frac{M}{15}$ solution, to produce a range of pH concentrations from 6.6 to 7.8. Tubes of these mixtures were heated to expel the air and to increase the

velocity of reaction. The solutions of a pH of 7.4, 7.6, and 7.8 were quickly decolorized. On cooling and exposure to air the blue color as promptly returned. Similar but less rapid effects were obtained with the 2 per cent peptone solution. The beef infusion appeared to be inert.

The reducing action of dextrose in alkaline solution is well known as the basis of the Fehling and Benedict tests in urine analysis. The same reaction is utilized here. The routine use of methylene blue in dextrose peptone broth is simply a convenient method of assembling the materials in a weakly alkaline solution.

The delicacy of the reaction depends upon the feeble reducing power of the medium at the chosen hydrogen ion concentration and at incubator temperature. An equilibrium is established between the rate of diffusion of oxygen through the medium and the activity of the reducing agent. It is only when the access of oxygen is practically completely inhibited that the weakly alkaline dextrose solution is able to fix the remaining traces of the gas and then to attack the methylene blue.

Comparison of Different Amounts of Methylene Blue.—From 0.1 to 1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of dextrose broth was decolorized with practically equal rapidity in less than 24 hours in a McIntosh and Fildes jar.[9] For the purposes of this study, variations in the small quantity of the dye used in the medium were of little significance.

Effect of Temperature.—Like other chemical reactions, the rate of reduction is a function of the temperature. When access of free oxygen is prevented decolorization does not occur in 24 hours at 4°C. It proceeds slowly at room temperature (21°C.) and rapidly at 37°C. and higher temperatures.

Estimation of the State of the Medium.—It has been noted above that the state of the indicator, whether blue or colorless, depends upon the relative rate of the admission and diffusion of oxygen from the air and the activity of a reducing substance, *e.g.* dextrose, in the medium. The equilibrium between these two forces may be observed in a narrow test-tube by the depth below the surface at which the colorless zone begins. While methylene blue may not be decolorized in an oxygen-free liquid in the absence of a reducing substance, decolorization indicates a strictly anaerobic condition, and the return of the color is an index of the return of oxygen to the medium.

---

[9] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

With these indications of the sensitiveness of the reaction and of the factors that control its progress, we proceeded with a study of the various elements in the technique under investigation.

Comparison of Liquid Paraffin Oil and Solid Vaseline as a Seal.

The tissue medium technique as generally employed involves the use of liquid paraffin oil as a seal or supernatant to the medium. It was presumed that the oil, besides preventing evaporation, favored deoxidation by opposing a barrier to the air. Rosenthal[10] suggested the use of lanolin (melting point 42°C.) as a seal, and recently Fildes[11] has stated that oil has practically no effect in preventing the return of oxygen to the medium, while solid paraffins merely delay its passage. In view of the importance of an effective seal, a quantitative comparison of oil and vaseline seemed advisable.

Experiment 1.—Twelve tubes, 1.5 by 20 cm., each containing one-sixteenth of the kidney of a 1,600 gm. rabbit, were filled with 1 per cent dextrose broth to a height of 9.5 cm.; 2 drops of sterile 0.5 per cent aqueous methylene blue were added as indicator. Series A consisted of four tubes, not sealed (air control). Series B, four tubes overlaid with 2.5 cc. of paraffin oil. Series C, four tubes overlaid with 2.5 cc. of vaseline. All incubated at 37.5°C.

When observed after 16½ hours the tubes of Series A were decolorized 2 cm. from the bottom of the tube. Tubes of Series B decolorized 3 cm. from bottom. Tubes of Series C completely decolorized.

When observed after 42 hours, the tubes of Series A and B showed an increase of 1 cm. in the height of the decolorized column. Tubes of Series C remained completely decolorized. There was no further increase in the height of the decolorized columns in the tubes of Series A and B.

Repetition with smaller fragments of kidney yielded similar results.

As shown by the point at which a balance was established between the access and diffusion of oxygen and the reducing action of the kidney tissue, the addition of paraffin oil produced only a slight increase in the length of the anaerobic column, the final ratio of the oil-covered to the air-covered tubes being 4:3.

[10] Rosenthal, G., *Compt. rend. Soc. biol.*, 1902, liii, 1132.

[11] Fildes, P., *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 12*, 1917, 59.

Experiment 2.—Sixteen 20 by 1.5 cm. tubes, filled with broth and methylene blue as before, but without kidney tissue. Series A, four tubes not sealed. Series B, eight tubes sealed with paraffin oil in a column from 0.5 to 4 cm. in height. Series C, four tubes, vaseline seal 1 cm. high. All were placed in a McIntosh and Fildes jar,[9] the oxygen was removed by combustion, and the tubes were incubated at 37.5°C.

After 18 hours Series A was completely decolorized. Series B, 0.5 to 1 cc. of oil, completely decolorized (1.5 to 2.5 cc. decolorized in 42 hours, 3 to 4 cc. in 60 hours). Series C, completely decolorized. Removal from jar and exposure to air caused a prompt return of color in the tubes of Series A and B. Series C remained colorless permanently.

The results of Experiments 1 and 2 are illustrated in Fig. 1.

In the McIntosh and Fildes jar a slight reduction in pressure occurs as a result of the combination of oxygen and hydrogen. The partial vacuum tends mechanically to lower the tension of oxygen dissolved in the medium and so hastens the establishment of strict anaerobic conditions through the action of a reducing agent. When large amounts of paraffin oil are employed, this favorable action is retarded. Indeed, the oil seems to serve as a reservoir for oxygen in solution. Except to prevent evaporation and to maintain anaerobic conditions after removal, the use of any seal appears to be unnecessary in a properly manipulated McIntosh and Fildes jar.

Experiment 3.—Six 20 by 1.5 cm. tubes containing 10 cc. of dextrose broth were colored with 1 drop of 1 per cent methylene blue. Series A, two tubes, unsealed. Series B, two tubes, overlaid with 4 cc. of paraffin oil. Series C, two tubes, overlaid with 2.5 cc. of vaseline. All tubes heated in a water bath until color disappeared, then plunged into cool water to bring to room temperature and to solidify the vaseline.

The blue color appeared immediately at the surface of the unsealed tubes and at the surface of the medium in contact with the oil. Streaks of blue carried by convection currents descended to the bottom of the tubes, there to be slowly decolorized again. After 20 minutes a zone of blue was established to a depth of 3.5 cm. in the unsealed tubes and to 3 cm. in the oil-covered tubes. The vaseline-covered tubes remained colorless.

After standing for 72 hours at room temperature the blue zone had descended to a depth of 4.7 cm. in the unsealed tubes and to 4.6 cm. in the oil-sealed tubes. During the following days the unsealed and the oil-sealed tubes came to an equilibrium approximately 6 cm. below the surface of the medium. The vaseline-sealed tubes remained colorless throughout.

Under air or paraffin oil, dextrose broth is unable alone to overcome the diffusion of oxygen, which is hardly retarded by the paraffin oil seal. In the depths of the tube, however, the combined reducing action of broth and kidney is able to establish and maintain an oxygen-free zone, which is slightly higher in the paraffin oil-covered tubes. Under vaseline, on the other hand, owing solely to its solid state at incubator temperature, the access of oxygen is prevented, and the dextrose and kidney tissue in the course of a few hours fix all the oxygen remaining in solution. Other aids to deoxygenation, such as a McIntosh and Fildes jar or the action of heat in reducing the oxygen solubility of the medium and increasing the chemical activity of the alkaline dextrose solution, exert a similar action in hastening the establishment of anaerobic conditions in a culture tube, provided that access of atmospheric oxygen is prevented by an impervious solid seal such as vaseline.

We conclude that the paraffin oil seal extensively used in anaerobic culture work and in gas analysis is practically valueless except to prevent evaporation. The seal itself may contain enough oxygen in solution to defeat the very object which it is used to attain. A layer of vaseline, on the other hand, is an oxygen-resisting seal that materially aids the action of deoxidizing agents in the medium.[12]

Standardization of the Kidney Tissue Component.

Four functions have been ascribed to the fragment of kidney utilized in the tissue technique: a reducing activity, the formation of a nidus, the contribution of nutritive elements to the medium, and an effect upon the pH concentration by acid production. That the kidney tissue component is an active reducing agent has been well known since 1885 through the researches of Ehrlich and others and is demonstrated anew in Experiment 1 of this communication. But the zone of its action in the culture tube has not been subjected to quantitative estimation.

[12] Hard, inelastic paraffin waxes may be less useful through rupture of their contact with the glass by changes of temperature in the tubes.

Smith,[1] who was the first to advocate the use of tissue fragments, gave no indication of the size to be used. Tarozzi[2] advocated a cube of about 1 cm. Noguchi[13] suggested that the kidney of an adult rabbit should be cut into about ten pieces, each approximately the size of a split chestnut. Other original workers have not defined the tissue component so carefully and the tendency in general has been to use too small a piece, especially for primary cultivations and early transplants.

Comparison of the Reducing Effect of Different Amounts of Kidney Tissue.—We tested the reducing effect of pieces of various sizes upon a medium containing methylene blue.

Experiment 4.—Two sets of three 20 by 1.5 cm. tubes were prepared, each containing 10 cc. of aerated 1 per cent dextrose broth and 1 drop of 1 per cent aqueous methylene blue in addition to the kidney fragments. The fragments were cut as uniformly as possible from 5 mm. cross-sections through the middle of the kidneys from an 1,800 gm. rabbit. In Set 1 a single piece of kidney was used. Tube A contained one-eighth, Tube B one-fourth, and Tube C one-half of a 5 mm. cross-section. In Set 2 the pieces were cut into eighths of the original cross-section. Tubes A, B, and C were the same as the corresponding tubes of Set 1, except that Tube B contained two-eighths and Tube C four-eighths of a cross-section in two and four separate fragments. No seal. Incubated at 37.5°C.

In Tubes B and C of each series decolorization began in a few moments around the kidney tissue at the bottom of the tube. Later results are shown in Table I and are illustrated in Fig. 2.

After 42 hours the tubes were removed from the incubator and allowed to stand at room temperature. After 20 hours at about 21°C. the level of decolorization had dropped 0.5 to 1 cm. in each tube.

TABLE I.

Comparison of the Reducing Effect of Different Amounts of Kidney Tissue.

Set No.	Duration of incubation.	Height of decolorized column from bottom of tube.		
		Tube A; small fragment of kidney.	Tube B; twice as much as Tube A.	Tube C; four times as much as Tube A.
	hrs.	*cm.*	*cm.*	*cm.*
1	16	0.4	2.3	3.0
2	16	1.0	1.8	3.0
1	42	1.7	3.5	4.4
2	42	1.8	3.4	4.4

[13] Noguchi, H., *Münch. med. Woch.*, 1912, lix, 1937.

Different amounts of kidney tissue have different reducing effects. The larger the amount, the greater is the reduction. Small pieces are of little value for producing an anaerobic zone. Very large pieces do not produce a proportionately large zone of reduction. With 0.6 to 0.8 gm. of kidney tissue a zone sufficient for practical purposes is obtained.[14] The tissue may be used in one fragment or in several. The balance between the penetration of oxygen from the surface into the medium and the reducing activity of the tissue occurs at a level determined by the temperature, other things being equal. The higher the temperature, up to 37°C., the less is the oxygen solubility and the greater the reduction.

Nature of the Reducing Substances in the Kidney Tissue.—Kidney tissue in unsealed tubes such as those of Experiment 4 exposed to the air at room temperature will maintain an anaerobic zone at the bottom of the tube for a period of weeks or months. It is not conceivable that the reduction is dependent on the maintenance of the activity of living cells. The question arises then, as to the nature of the substance responsible for the reduction. In considering this question, which properly belongs to physiology and biochemistry, we were soon led away from the subject in hand. Preliminary experiments indicated that the activity still resided in filtered extracts of kidney tissue, and that the reducing substance concerned is relatively heat-stable, so that boiled, or even autoclaved kidney is not entirely without effect, but other considerations make it seem unprofitable to attempt to modify the use of fragments of fresh sterile kidney tissue in this anaerobic technique. Thunberg[15] refers to former researches on the reducing activity of tissues and reports his evidence of the enzymotic nature of the reaction.

Before the reducing activity of fresh tissue in anaerobic cultivation was generally recognized, it was suggested that the tissue fragment might act in an obscure and passive manner as a focus for bacterial multiplication. The demonstration of a favorable reaction on the

[14] The kidneys of a medium sized rabbit, 1,400 to 1,700 gm., weigh about 6 to 6.5 gm. Eight to ten fragments may be obtained from each kidney. The large kidneys of full grown rabbits yield twelve to sixteen fragments of proper size.

[15] Thunberg, T., *Skandin. Arch. Physiol.*, 1918, xxxv, 163.

surrounding medium made such an assumption unnecessary. Some investigators, however, acting on the earlier hypothesis, have advocated the use of small pieces of inorganic substances, asbestos wool or iron tacks, for example,[16] as a nidus for anaerobic growth.

Our experiments with these substances in the presence of methylene blue need not be elaborated here. They indicate that washed asbestos wool has no effect in promoting an anaerobic zone. Indeed, in a feeble reducing medium the decolorization of the methylene blue was somewhat retarded. The oxidation of iron tacks, on the other hand, soon reduced the dye in their vicinity. It seems clear that the production of an anaerobic condition depends upon a chemical reaction rather than upon the presence of inert material.

Nature of the Culture Medium.

Presence of a Reducing Substance.—Methylene blue serves as an indicator of the presence or absence of free oxygen only in the presence of a reducing substance. For this reason it is not an accurate index for the removal of oxygen by physical means. Our observations led us to conclude, however, that complete deoxygenation is only very slowly accomplished by the diffusion of oxygen in solution into an oxygen-free atmosphere such as is provided by the method of Buchner[17] or that of McIntosh and Fildes.[9]

In a recent communication Barber[18] indicates that some strict anaerobes may be destroyed even by short exposures—less than an hour—to atmospheric oxygen. Under certain conditions, therefore, it may be important to attain strictly anaerobic conditions in the culture tube in the shortest possible time after inoculation or else to inoculate an already deoxygenated medium.[19] Among the aids to such a procedure is the addition of an active reducing agent (kidney tissue, dextrose, peptone) to the culture medium.

Culture media in general may be divided into two classes, those which contain an active reducing substance, and those which are prac-

[16] Douglas, S. R., Fleming, A., and Colebrook, L., *Lancet*, 1917, ii, 530.

[17] Buchner, H., *Centr. Bakt.*, 1888, iv, 149.

[18] Barber, M. A., *J. Exp. Med.*, 1920, xxxii, 295.

[19] Tarozzi[2] and Noguchi[5] undoubtedly obtained such a condition by preliminary incubation of tissue media to insure sterility.

tically inert. Artificial media containing dextrose or peptone belong in the first category. Ascitic fluid and dilute serum, widely used in anaerobic culture work, belong in the second class. Although small amounts of a copper-reducing substance may be demonstrated in these liquids, their deoxygenating action is relatively slight, and hardly to be considered of practical value. Thus, while kidney tissue was able to decolorize 10 cc. of ascitic fluid containing methylene blue under a vaseline seal in 10 days, similar tubes of ascitic fluid, without kidney tissue, still retained a pale blue color after 6 weeks observation.

The efficacy of dextrose as a reducing agent in alkaline solution suggested its addition to the ascitic fluid medium. Combinations of 0.1 to 2 per cent of dextrose were made by the addition of 10 per cent dextrose solution in isotonic saline solution to the ascitic fluid. Vaseline-sealed tubes containing ascitic fluid and methylene blue and 0.1 per cent dextrose were almost decolorized in 7 days. The larger amounts of dextrose up to 2 per cent, with the aid of a McIntosh and Fildes jar, decolorized the ascitic fluid in 5 to 6 days. Dextrose peptone broth was even more efficacious. One part of 1 per cent dextrose peptone broth with two parts of ascitic fluid decolorized methylene blue under a vaseline seal in less than 42 hours.

In the absence of a reducing agent tubes of ascitic fluid from which air is rigidly excluded may not become decolorized over a period of weeks. The addition of small amounts of dextrose, or the presence of kidney tissue renders ascitic fluid medium oxygen-free in a relatively short time. It should be understood that dextrose here is being considered solely as a reducing agent. Other effects—change of pH concentration through bacterial action, gas formation, etc.—may make its addition undesirable in certain instances.

Physical State of the Medium.—A second character of culture medium which influences the establishment of anaerobic conditions is its physical state. Fluid media suffer the disadvantages of ready diffusion of oxygen throughout the tube, of the development of convection currents on even slight changes of temperature, and of the transfer of oxygen to the depths of the tube by any agitation. These phenomena are easily observed in air or oil-covered tubes showing a decolorized zone at the bottom.

Semisolid media, formed by the addition of small amounts of agar, are not subject to these influences to the same extent. It is not surprising, therefore, to find that anaerobic conditions are more readily maintained in the depths of a semisolid culture tube. Thus, the effect of different amounts of agar on the diffusion of oxygen through dextrose broth was tested.

Experiment 5.—Tubes were filled with 1 per cent dextrose peptone broth and 2 per cent dextrose agar in proportions to give percentages of agar from 0 to 0.5 in volumes of 10 cc. The mixtures were colored with methylene blue and heated in the water bath to complete decolorization. They were then plunged into water to solidify the agar and incubated at 37°C. The diffusion of oxygen from the surface downward was measured in all the tubes by the advance of the returning color. After 5 and 24 hours the blue zones had descended as shown in Table II, where they remained during subsequent observations.

TABLE II.

Penetration of Oxygen into Semisolid Medium.

Tube No.	Amount of agar.	Penetration of oxygen from surface of medium.		
		After 5 hrs.	After 24 hrs.	After 48 hrs.
	per cent	*cm.*	*cm.*	*cm.*
1	0	1.0	4.0	Complete.
2	0.02	1.0	1.5	3.3
3	0.04	0.9	1.2	2.2
4	0.06	0.9	1.1	1.3
5	0.1	0.8	1.0	1.1
6	0.2	0.8	1.0	1.1
7	0.5	0.7	0.9	1.0

Even so small an amount as 0.02 per cent of agar may inhibit the diffusion of oxygen to the depths of a culture tube, or at least so retard it that dextrose broth is able to maintain anaerobic conditions below a certain level. This level occurred at 1 cm. from the surface when 0.5 per cent of agar was employed.

A more striking experiment is afforded by a comparison of the reducing effect of kidney tissue in peptone broth and in the same broth made semisolid by the addition of 0.25 per cent agar.

Experiment 6.—Series A; tubes contained a 9 cm. column of plain broth. Series B; the same plus kidney tissue. Series C; tubes contained a 9 cm. column of semisolid medium (peptone broth plus 0.25 per cent agar). Series D; the same plus kidney tissue. All were colored with methylene blue and were incubated unsealed at 37.5°C. Table III shows the height of the colorless zones which developed from the bottom of the tube in each instance.

TABLE III.

Development of Anaerobic Conditions by the Action of Kidney Tissue in Fluid and Semisolid Medium.

Medium.	Zone of decolorization measured from bottom of tube.		
	After 1 day.	After 2 days.	After 6 days.
	cm.	*cm.*	*cm.*
Series A; plain broth without kidney tissue	0	0	0
" B; " " with kidney tissue	1.5	2.0	1.4
" C; semisolid medium without kidney tissue	7.4	7.3	7.3
" D; " " with kidney tissue	7.0	7.3	7.3

Besides showing that oxygen penetrates slowly into semisolid media this experiment demonstrates the reducing power of peptone (Series C). Comparison of Series C with Series D suggests that the reducing power of kidney tissue in a semisolid medium is confined to the zone surrounding the tissue, since its effect did not extend further than that of the peptone broth alone.

This observation is confirmed by the following experiment with a medium of much less reducing power, so that the kidney tissue alone was responsible for the production of an anaerobic zone.

Experiment 7.—Series A, two tubes, each containing one-tenth of a medium sized kidney, 7.5 cc. of dilute rabbit serum, 2.5 cc. of 2 per cent plain agar, and 1 drop of methylene blue, making a 0.5 per cent semisolid medium. Series B, two tubes, each containing the same ingredients, except that 2.5 cc. of plain broth were substituted for the agar, making a fluid control. Control tubes of each medium, without kidney tissue, were also set up. Vaseline seals. Tubes incubated at 37.5°C. for 24 hours. The tubes of Series A showed a decolorized zone 1.7 and 1.7 cm. high respectively, sharply demarcated from the deep blue agar above. Control, without kidney, deep blue. The tubes of Series B were decolorized 2.4 and 2.3 cm. from the bottom, respectively, shading off gradually to a deep blue above. Control, without kidney, deep blue. After 48 hours the semisolid tubes showed a clear-cut colorless zone, 2.7 and 2.6 cm. high; agar deep blue above. Control

deep blue. The fluid tubes showed a colorless zone approximately 4 to 4.5 cm. high, shading off so gradually that its limits were difficult to determine. The fluid above and the control were light blue.

From these experiments a semisolid medium is seen to favor the establishment of anaerobic conditions by the exclusion of oxygen from the depths of the tube. Unless a reducing agent is diffused through the medium, however, this advantage is offset by a restriction of the reducing power of the kidney tissue to the immediate vicinity of the tissue fragment. These observations may explain in part the difficulty often encountered in growing strict anaerobes in semisolid medium, especially in initial cultivation.

Length of the Column of Medium as an Aid in Deoxygenation.—It has become the standard practice in anaerobic cultivation by the tissue method to use 12 to 15 cc. of fluid, or a column 10 to 12 cm. in height in a long narrow culture tube 20 by 1.5 cm. Anaerobiosis in the depths of the tube would seem to be favored by the restricted surface exposed under paraffin oil and by the length of the column of liquid.

It was found in the present study that in a reducing medium such as dextrose broth a column length of from 8 to 16 cm., overlaid with paraffin oil, favors the decolorization of methylene blue, which occurs first at the bottom of the tube and gradually extends toward the surface. Under vaseline, on the other hand, columns of different lengths decolorize with equal rapidity. In dextrose broth the reducing action proceeds throughout the tube. When it is more localized, as with kidney tissue in a less active medium (plain broth), the effect is somewhat different.

Experiment 8.—Three sets of tubes of plain broth colored with methylene blue, each containing one-sixteenth of a kidney of a large rabbit, the broth columns being 2, 4, 6, 8, 10, and 12 cm. long. Series A, unsealed; Series B, overlaid with 2 cm. of oil; Series C, overlaid with 1 cm. of vaseline. Control tubes of broth without kidney, column 8 cm. long, unsealed and also with oil and vaseline seals. Incubated at 37.5°C. In 16 hours decolorization had proceeded as shown in Table IV.

When oxygen is excluded (Series C) the fragment of kidney tissue is able to produce an anaerobic zone 2.1 cm. high in 16 hours. That

it did not do so in the 2, 4, and 6 cm. tubes of Series A and B under air or oil is due to the penetration of oxygen. A column of fluid 8, 10, or 12 cm. long maintained at a constant temperature without agitation practically serves as a seal for the lower levels. But the same object is accomplished by a vaseline seal with considerable saving in medium; 7 or 8 cm. of the medium suffice.

TABLE IV.

Effect of the Height of the Medium Column on the Anaerobic Zone Produced by Kidney Tissue.

Series.	Height of decolorized column from bottom of tube after 16 hrs.						Control.
	Length of fluid column.						
	2 cm.	4 cm.	6 cm.	8 cm.	10 cm.	12 cm.	8 cm.
	cm.	*cm.*	*cm.*	*cm.*	*cm.*	*cm.*	
A	1.0	1.5	1.8	2.2	2.1	2.1	Unchanged.
B	1.3	1.8	1.9	2.1	2.0	2.1	"
C	2.0	2.1	2.0	2.1	2.1	2.1	"

Effect of Reducing Agents on the pH of the Medium.

Although this study deals only with the deoxygenation of culture media, it is necessary to know whether the reducing agents employed have any effect upon other essential factors in anaerobic cultivation; for example, the hydrogen ion concentration of the medium. Accordingly, tubes were filled with ascitic fluid or with ascitic fluid and dextrose peptone broth, with or without kidney tissue. To each tube 5 drops of phenol red were added and the tubes incubated at 37.5°C. under a vaseline seal. When observed at intervals during the following days and weeks, the tubes without kidney tissue showed no change from the original hydrogen ion concentration of 8 +. On the other hand, the kidney tissue in 20 hours had produced a clear yellow zone approximately 1.5 cm. from the bottom of the tube. The effect was the same in ascitic fluid and in the mixture of ascitic fluid and broth. The next day and thereafter, all the tubes containing kidney tissue showed a gradation from purplish pink (pH 7.8 to 8) at the surface downward in the acid direction to a clear yellow in the middle and lower portions of the column of medium. This color

gradually became diffused throughout the tubes, bringing the medium to an orange-yellow comparable to pH 7.4 on the phenol red scale.

Ascitic fluid itself is usually alkaline, showing a pH 7.8 to 8+. This alkalinity is progressive if the fluid is allowed to stand exposed to the air but may be retarded by a layer of paraffin oil. From the foregoing experiment it is seen that the alkalinity, once established, remains unaltered for weeks under a vaseline seal. Dextrose and peptone do not in themselves produce acid and thus change the hydrogen ion concentrations. Only when they are split by bacterial activity do they have this effect. Kidney tissue produces acids; a large fragment (0.8 gm.) changes the hydrogen ion concentration in its immediate vicinity to about pH 7. This acid becomes gradually diffused throughout the medium and may ultimately bring the entire column of medium to a favorable hydrogen ion concentration (pH 7.4). The final concentration, then, depends on the ratio of the original alkalinity of the ascitic fluid to the acid production of the kidney.

SUMMARY AND CONCLUSIONS.

This study was undertaken with the object of determining the part played by the several component elements of the tissue method of anaerobic cultivation in the establishment of anaerobic conditions in the culture tube. Data have been presented to show the suitability of methylene blue as an indicator of reduction processes in culture media by which the removal of the last traces of oxygen may be demonstrated. With methylene blue as the indicator, the elements subjected to experiment included the choice of a seal for culture tubes, the activity and requisite size of the kidney tissue fragment, the chemical and physical characters of the medium which promote or retard deoxygenation, the length of the column of medium, and the advantages of external aids such as the McIntosh and Fildes anaerobic jar.

As a result of our experiments, we have come to the following conclusions:

1. Liquid paraffin oil, used extensively as a seal for anaerobic cultures and in gas analysis, has very little value in inhibiting the access of oxygen. Solid vaseline, on the other hand, forms an effective

oxygen-resisting seal. The difference is due to the physical states of the substances at incubator temperature.

2. Fresh kidney tissue is an active reducing agent and quickly decolorizes methylene blue in its vicinity. The reducing effect of fresh kidney tissue is relative to the amount used. As a reducing agent, at least 0.6 gm. per tube is required for the establishment of an adequate oxygen-free zone.

3. Culture media may be classified as reducing or non-reducing. Those containing dextrose or peptone in a faintly alkaline solution belong to the former class. Ascitic fluid and dilute serum belong to the latter class, for their content of reducing substances is practically insignificant. For the prompt establishment of strictly anaerobic conditions these media require the addition of reducing substances such as dextrose, peptone, or kidney tissue aided by an effective seal or an anaerobic jar.

4. Semisolid media effectively inhibit the penetration of oxygen to the depths of the tube, but they likewise limit the diffusion of reducing substances and presumably of nutrient substances from imbedded kidney tissue.

5. The length of the column of medium is of minor importance under a vaseline seal.

We clearly recognize the impracticability of standardizing a biological technique which by its very nature must be subject to wide modifications for special purposes. Such variations from a standard are especially necessary in the search for unknown organisms, and in work with hitherto uncultivated microbes in which the tissue technique has been successfully applied by Noguchi.

We wish, therefore, to present the results of our studies simply as guides in the variation and control of the elements examined and to make certain suggestions relative to the establishment of strictly anaerobic conditions in the culture tube. The numerous other factors of equal importance which must be taken into account—hydrogen ion concentration, source and character of nutritive elements, temperature, time, etc.—are outside the limited scope of the present report.

For the establishment of strictly anaerobic conditions in the culture tube, we would suggest (1) the substitution of solid vaseline for liquid paraffin oil as an oxygen-resisting seal; (2) the use of large pieces

of fresh kidney, the standard size to be upwards of 0.6 gm. unless other reducing substances are present in the medium; (3) the addition of peptone or dextrose or both in the form of peptone dextrose broth in fractional percentages to non-reducing media such as ascitic fluid or serum to aid in the prompt establishment of anaerobic conditions; (4) the use of the McIntosh and Fildes jar as a further aid to the prompt deoxygenation of the medium; (5) for reasons of economy the use of smaller amounts of culture medium, for example, 7 to 8 cc., under a vaseline seal; and (6) in dealing with anaerobes which may be injured by exposure to oxygen it might be advisable to prepare the medium a day or two in advance and to incubate it under a vaseline seal so that sterility is assured and the anaerobic conditions are already established when inoculation is made. The infected material is then introduced with a capillary pipette in the vicinity of the kidney tissue and the seal restored by gentle heating to melt a portion of the superposed vaseline.

EXPLANATION OF PLATE 2.

FIG. 1. A comparison of paraffin oil and vaseline as oxygen-resisting seals. Sample tubes from Experiments 1 and 2. Dextrose broth and methylene blue without and with kidney tissue, unsealed and under paraffin oil and vaseline. Tubes 1, 3, and 5, without kidney, were deoxygenated by the dextrose broth in a McIntosh and Fildes jar. On removal from the jar the color has returned in the unsealed and oil-covered tubes (Nos. 1 and 3), denoting the penetration of oxygen into the medium. The vaseline-covered tube (No. 5) remains colorless.

Tubes 2, 4, and 6, with kidney, show its reducing effect in the depths of the tube. The unsealed broth (Tube 2) shows almost as extensive a zone of decolorization as the oil-covered broth (Tube 4). Aided by complete exclusion of oxygen, the kidney and dextrose broth have completed the deoxygenation of the vaseline-covered tube (No. 6).

FIG. 2. A comparison of the deoxidizing value of kidney tissue fragments of different size. *A*, a fragment of the size ordinarily employed. *B*, a fragment twice the size of *A*. *C*, a fragment four times the size of *A*. The establishment of an oxygen-free zone is denoted by the decolorization of the medium.

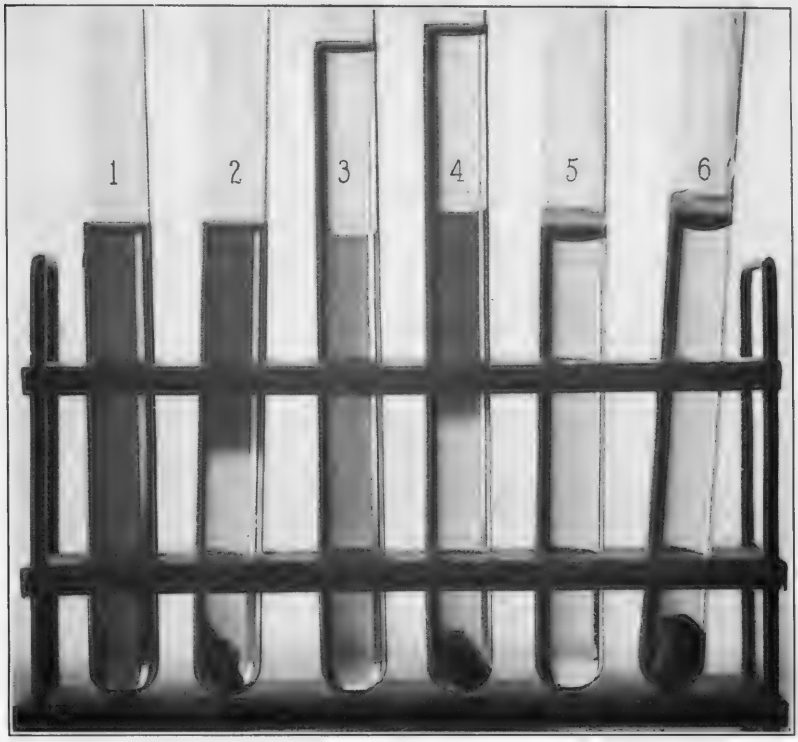


FIG. 1.

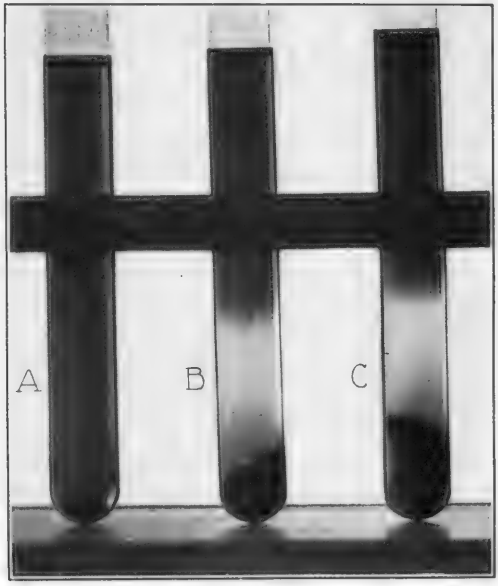


FIG. 2.

(Gates and Olitsky: Factors influencing anaerobiosis.)

PRESERVATION OF STOCK CULTURES OF BACTERIA BY FREEZING AND DRYING.*

By HOMER F. SWIFT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, June 29, 1920.)

The object of this communication is to present the results of the prolonged preservation of stock cultures of bacteria after freezing and drying, and to describe the method used.

The benefit of a method that will assure the preservation of cultures of bacteria without frequent manipulation is self-evident. As type organisms are isolated or identified it is desirable to keep the original state of virulence or to retain their cultural characteristics. In addition to the danger of contamination from frequent manipulation, there is the well known tendency for many bacteria to lose their virulence or other biological characteristics from repeated transfers on artificial media. During the course of many investigations it is often of advantage to keep all the bacteria isolated until there is time for more detailed study. A method that will permit the shipping of cultures from one city or country to another without the bulk of culture media, or without danger of breakage or death of the bacteria, has obvious advantages.

The principle of keeping bacteria in a dormant state by desiccation after freezing has been known for several years. Shackell (1) was one of the first to call attention to its advantages. He showed, first, that after freezing, substances such as tissue could be uniformly dried; second, that serum retained its complement and antibody activity unaltered for weeks; and third, that rabies virus in rabbit brains did not lose its virulence when kept in this manner. Hammer (2) dried bacteria previously frozen on strips of filter paper, and kept them from 54 to 57 days. Controls, dried without freezing, were killed immediately. Shattock and Dudgeon (3) showed that organisms dried on charcoal without freezing were usually killed in from 4 to 40 days. *B. pyocyaneus*, however, lived for at least 7

* Read before the American and Canadian Section of the International Association of Medical Museums, New York, N. Y., April 1, 1920.

months. Macfadyen (4) and Macfadyen and Rowland (5) demonstrated that practically all bacteria as well as yeast could be frozen and reduced to a temperature of liquid hydrogen, *i.e* −252°C., and later recovered in a viable and unchanged condition. Rogers (6) finally applied the principle of freezing and drying on a large scale so that mass cultures of lactic acid-forming bacilli could be preserved for commercial purposes. He suggested the use of this method for the preservation of stock cultures for the laboratory.

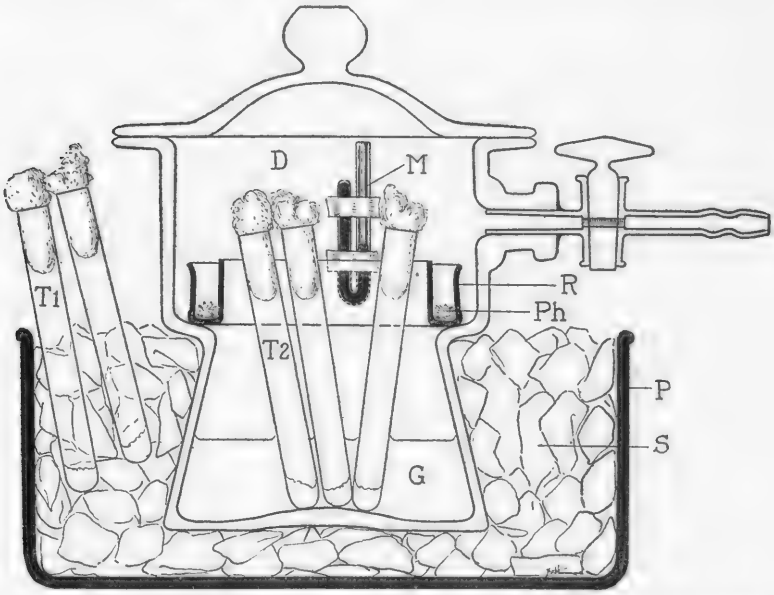
For the past 6 years we have been using this method in the preservation of stock cultures of streptococci and pneumococci. Recently upon attempting to recover these bacteria it was found that if they were originally prepared under proper conditions they were viable after a period of from 2 to 4 years. Comparison of the tubes from which the organisms could not be recovered, with those from which they were easily recovered, showed that the physical state of the dried material had much to do with the viability of the organisms. For example, of twenty-two tubes in which it was possible to recover bacteria, nineteen were in a condition of "dry foam" and three in a condition of "flaky gum." Among eleven with which failure occurred, nine were distinctly gummy, one showed a "flaky gum," and one a "dried foam." This physical state with the accompanying success or failure was present immediately after the organisms were dried or after 4 years. The results showed that it was necessary to maintain the frozen state until drying was complete; this led to the devising of the present method in which the tubes are immersed in glycerol which acts as a medium for the conduction of cold from a salt-ice mixture.

Method.

Apparatus (*Text-Fig. 1*).—A desiccator (*D*) is chosen with broad opposing surfaces on cover and stop-cock. It is usually necessary to regrind all opposing glass surfaces. On the inner side of the desiccator is fastened with adhesive plaster a small mercury manometer (*M*) with one open and one closed end. In the upper part of the desiccator is a receptacle (*R*) for the desiccating salt; if phosphorus pentoxide (*Ph*) is used this container can be of copper. In the bottom of the desiccator is placed about 4 cm. of commercial glycerol (*G*). A pan (*P*) is provided, large enough to hold the desiccator and

a salt-ice mixture (*S*) which is packed around the latter. It is necessary to have a mechanical pump that will give a vacuum as low as 2 or 3 mm. of mercury; preliminary exhaustion of the larger part of the air can be conveniently carried out with the ordinary water pump.

Preparation of Cultures.—The object to be attained is a maximum number of young actively growing forms of the bacteria in a minimum



TEXT-FIG. 1. Diagrammatic cross-section of the apparatus used for freezing and drying bacterial cultures for preservation. *D*, desiccator; *M*, mercury manometer; *R*, copper receptacle for the phosphorus pentoxide (*Ph*); *G*, glycerol in the bottom of the desiccator; *P*, pan for the salt-ice mixture (*S*); *T 1*, position of a tube of culture during freezing; *T 2*, position of a tube of culture during desiccation.

of fluid. It is necessary to have enough fluid to form a small amount of ice. If fluid media are used it is well to grow the organisms for 15 to 18 hours, centrifugalize the culture, and discard most of the supernatant broth. The concentrated bacteria can then be pipetted into a number of small tubes and frozen. With certain bacteria it

is necessary to use the growth from solid media. In this case the growth should be scraped off into a small amount of condensation water or of broth that has been added, and the suspension subsequently pipetted into tubes. We have found it convenient to use small tubes, about 10 cm. long and from 5 to 10 mm. in diameter.

Manipulation.—After the culture has been placed in the small tubes as above described the desiccator containing the glycerol is set in the pan containing the salt and ice mixture; the tubes as well are immersed in this mixture and the cultures frozen (position *T 1*, Text-fig. 1). Simultaneously the temperature of the glycerol is reduced to minus 4–6°C. After careful lubrication of the desiccator cover and stop-cock and after the glycerol has reached a proper temperature and the bacterial suspension is well frozen, the tubes are immersed in the glycerol (position *T 2*), the phosphorus pentoxide is put in the copper pan, and the cover placed securely on the desiccator. The air is then exhausted from the desiccator, first with an ordinary water pump and finally with the high vacuum pump, during all this time the desiccator being kept in the salt-ice mixture. When the proper degree of vacuum has been obtained the whole apparatus is placed in the ice box where it is left until desiccation is complete.[1] This time, in our experience, is usually about 12 hours. When it is certain that desiccation is complete the tubes are removed from the desiccator, the cotton stoppers pushed down into them, and melted paraffin is poured in until the tubes are thoroughly sealed. It is usually necessary to paraffin the tubes two or three times in order to insure complete sealing. If it is desired to keep the organisms for many years the tubes may be sealed by melting and fusing the open ends of the tubes. After the tubes have been well labelled, they can be kept at room temperature, preferably in the dark.

The appearance of the properly dried culture is that of a very light, spongy, flaky material. If it is separated from the sides of the tube it can be shaken about and looks like dried lather made from shaving soap.

Recovery of Organisms.—The organisms may be recovered in one of several ways. For such bacteria as the cocci, and those of the

[1] If a frigo ice box is available it is advisable to place the desiccator at a temperature below zero.

colon, typhoid, and dysentery group, as well as other organisms that grow easily in broth, after the removal of the stoppers, broth may be added directly to the dried powder and the tubes incubated. Organisms like meningococci or others that grow better on solid media are best recovered by picking up a small amount of the dried material on a platinum loop and smearing it over the surface of suitable media, after it has been moistened in the water of condensation.

Results obtained by this method with various types of organisms are given below.

Streptococci and Pneumococci.—Out of fifteen strains of non-hemolytic streptococci kept from 22 to 40 months, all except two showed growth like that of the original culture, with similar fermentation reactions. These two, however, showed similar types of fermentation reactions with all the subcultures recovered; it is therefore probable that the change in fermentation occurred before the organisms were frozen. In the strains which originally showed a moderate degree of virulence, the virulence was maintained after the recovery of the organism. Four strains of hemolytic streptococci have been recovered after a period of from 42 to 51 months. Three strains of pneumococci were preserved in this manner and upon recovery several months later had retained their original virulence and reaction to type serum. One of the strains that had been accustomed to grow on 75 per cent bile retained this property in the subcultures.

Meningococci.—It has been found best to grow meningococci on solid media, scrape off the growth in a small amount of water of condensation, and freeze the resulting suspension. An experiment was performed to determine how small an amount of meningococci might be preserved in this manner; it was found that one loopful suspended in 0.2 cc. of broth or condensation water could be kept for a period of at least 2 months following desiccation. No tests were made after longer intervals. In the recovery of meningococci it has been found much more satisfactory to smear the dried powder upon the surface of freshly prepared blood agar or dextrose serum agar; the addition of blood dextrose broth to the dried powder has yielded more uncertain results. *Bacillus influenzæ* is best treated in the same manner as meningococci.

Other Organisms.—Typhoid, paratyphoid, and dysentery bacilli have been frozen following growth either in broth or on solid media. They were easily recovered by adding broth to the dried culture.

Other organisms of the bacteria group have not been tested because it is felt that those mentioned above represent the various types. There are few bacteria more delicate and more difficult to maintain in stock cultures than the meningococci and *Bacillus influenzæ*. Attempts have been made to preserve the spirochete of relapsing fever recovered from the blood of rats inoculated with these organisms, but the freezing seems to be sufficient to kill completely all the organisms. It is probable that spirochetes in general are not susceptible to preservation in this form, as a number of different strains have been shown to succumb to freezing. No work has been carried out by us with filterable viruses, but Harris and Shackell (7) have shown that the virulence of rabies virus is retained in this manner. Rous[2] has been able to preserve the virus of chicken sarcoma by a similar method for 7 years.

SUMMARY.

Attention is called to the fact that bacteria may be preserved for a long time by desiccation in the frozen state. It has been shown that it is necessary to maintain the frozen condition until desiccation is complete; if the fluid melts before the moisture is completely removed, the organisms are killed, probably because of the concentration of the salts upon the surface of the bacteria. By the simple expedient of immersing the tubes of organisms in glycerol contained in a desiccator and subsequently keeping the whole apparatus in a salt-ice mixture until drying is complete, the organisms are easily maintained in the frozen state, and dry properly. Bacteria preserved in this manner retain their cultural, biochemical, and immunological characters for prolonged periods.

[2] Rous, P., personal communication.

BIBLIOGRAPHY.

1. Shackell, L. F., *Am. J. Physiol.*, 1909, xxiv, 325.
2. Hammer, B. W., *J. Med. Research*, 1911, xxiv, 527.
3. Shattock, S. G., and Dudgeon, L. S., *Proc. Roy. Soc. London, Series B*, 1912, lxxxv, 127.
4. Macfadyen, A., *Proc. Roy. Soc. London, Series B*, 1900, lxvi, 180.
5. Macfadyen, A., and Rowland, S., *Proc. Roy. Soc. London, Series B*, 1900, lxvi, 488.
6. Rogers, L. A., *J. Infect. Dis.*, 1914, xiv, 100.
7. Harris, D. L., and Shackell, L. F., *J. Am. Pub. Health Assn.*, 1911, i, 52.

QUANTITATIVE DISTRIBUTION OF PARTICULATE MATERIAL (MANGANESE DIOXIDE) ADMINISTERED INTRAVENOUSLY TO THE CAT.

By CECIL K. DRINKER, M.D., and LOUIS A. SHAW.

(*From the Laboratory of Applied Physiology of Harvard Medical School, Boston.*)

Plates 3 and 4.

(Received for publication, June 26, 1920.)

INTRODUCTION.

The distribution of particulate material introduced intravenously into animals is a subject which has been approached from widely different points of view during the last 75 years. There is, first, a large literature concerning questions of lymph formation, capillary structure, and permeability. Thus, such easily recognized objects as mercuric sulfide particles, milk droplets, etc., have been followed microscopically in their passage from blood to lymph, and numerous efforts have been made to delineate the blood capillaries and lymphatics in organs such as the liver, where their structure is still a matter for debate. With the rise of histological pathology and still more with the growth of bacteriology, similar intravenous injections have been made with the idea of picturing the sites of lodgment of infecting microorganisms. In recent years, under the impetus of investigations by Bouffard (1), Goldmann (2), and others upon the distribution of certain colloidal dyes, many different types of injection in which the individual particles are visible microscopically have been employed. In these instances certain cells which are easily identified by their tendency to take up dye molecules have been shown to possess power to phagocytose visible particulate material as well.

A further cause for the complexity and extent of the literature is found in the uncertainty as to whether bacteria, living or dead, exhibit initial points of lodgment similar to those of inert particulate material such as carbon and mercuric sulfide.

All these questions result in an extensive and confusing literature, which, while it discusses in qualitative terms many important histological and pathological points, is lacking in quantitative data. Thus Ponfick (3), in 1869, injected suspensions of mercuric sulfide intravenously into the frog, dog, rabbit, and guinea pig. The amount of injection used is not made clear. There are no data as to the size of the individual particles, whether they are discrete or tend to agglutinate—in short, nothing which permits judgment upon the percentage of the injected material deposited in any particular organ, not as a result of embolism,

but as a result of fundamental properties on the part of the organ in question. The experiments of Ponfick (3) depend upon histological methods for determination of the distribution of mercuric sulfide, and while such methods must still be used in order to demonstrate the position in any particular organ which deposited material occupies, they are of practically no value in demonstrating the actual extent of deposition for each organ.

With the exception of the work of Voigt (4), who employed colloidal silver and silver iodide injections containing particles of ultramicroscopic size, there is a decided lack of information upon quantitative distribution of particulate material until the period of experiments with bacteria is reached. This deficiency arises from two causes: first, carbon, mercuric sulfide, carmine, fat droplets, etc., are not capable of being determined quantitatively in tissue except with difficulty and at best inexactly; and, secondly, the investigators who have employed such agents have been interested in histological deposition within particular organs and cells rather than in the exact amounts distributed in organs throughout the body.

If we consider, first, the question of inert particulate material such as carbon, we find it generally held that intravenously administered suspensions in the mammal lodge immediately in the liver, spleen, lungs, and bone marrow. In every instance the impression of the investigator as to the amounts reaching each organ is gained from histological preparations, and in order to make these preparations vivid the injected animals are flooded with particles. Under such circumstances one may report that certain groups of cells are extremely active in engulfing particles and may assign them an origin, but the experiment does not lead to conclusions as to the normal activity of the different organs in clearing the circulating blood of foreign particles, nor does it give reliable information as to the extent to which the organs involved engage in this activity.

It is thus clear that important information in regard to the deposition and fate of particulate material in the mammalian body must unfailingly result from the employment of a substance possessing certain attributes. The material must be insoluble in the body fluids and unagglutinated by them, capable of fine subdivision and suspension, and the suspensions employed must have been so prepared as to contain no particles or aggregates of particles capable of blocking capillaries. The material must be visible microscopically, and, most important of all, it must be susceptible to accurate and rapid chemical quantitative estimation even if in extremely high dilution. During the past year Reiman and Minot (5) have developed a method for the quantitative determination of manganese which fulfills the chemical requirements of the situation. In manganese

dioxide, with which this communication deals, and also with other compounds of manganese to be reported upon at a later date, we have found material which meets all the requirements necessary for injection experiments. It has proved possible with manganese dioxide to measure the rate of disappearance from the circulating blood, to determine the amount removed by different organs, and, lastly, to correlate these findings with histological observations which, with this compound, are satisfactory, since the particles are as black and as distinctive as carbon.

EXPERIMENTAL.

Manganese Dioxide Suspension.

The suspensions used for injection have been made from one 5 pound bottle of J. T. Baker's powdered manganese dioxide, chemically pure (84.5 per cent). 10 gm. of manganese dioxide are thoroughly shaken with 200 cc. of distilled water, centrifugalized several times at high speed, and the supernatant liquid is discarded until the particles have been thoroughly washed and the finest material has been largely eliminated.

The next step is to eliminate, by slow centrifugalizing, the larger particles, and in particular the clots composed of aggregates of small particles. This slow centrifugalizing is effected in a 0.4 per cent acacia solution, the acacia serving to hold up the finer particles and thus rendering the selective process of the centrifuge more effective. The acacia used for the purpose is taken from a 10 per cent solution to which 0.1 per cent sodium hydroxide is added until the solution is just alkaline to phenolphthalein. The supernatant liquid from this period of centrifugalization is saved and the residue discarded, since it contains the particles which are large enough to cause embolism.

In the numerous different suspensions used by us during the past year care has been taken not to employ those containing particles or aggregates of particles larger than 1μ. The experimental data which follow record deposition of manganese dioxide in milligrams of manganese. While the suspensions employed permit enumeration of the individual particles, it is cumbersome to record deposition in

individual organs in terms of number of particles, nor is it essential to do this provided care has been taken to keep the injected material uniform. The actual number of particles in the suspensions per milligram of manganese has varied between 2.5 and 5 billion.

The final suspension, in a 0.4 per cent acacia solution, is sterilized and put up in bottles for future use. Experience has demonstrated the fact that a suspension of manganese dioxide cannot be preserved free from aggregates of particles unless it stands in 0.4 per cent acacia solution and is sterile. Before making an injection the suspension is made slightly saline to the extent of 0.5 per cent sodium chloride, but under no condition should the suspension containing sodium chloride be allowed to stand over a period of hours, since even with acacia present there is a tendency for the particles to aggregate. This tendency is not great, the stabilizing power of even small traces of acacia being pronounced, but since it seems advisable to make injections in which the particles are as nearly absolutely discrete as pos sible, we emphasize the above precaution.

In order to prove that manganese dioxide is insoluble in blood we attempted to determine whether the compound used could be found to have dissolved when it was exposed to the action of sterile blood at 38°C. Owing to difficulties with filtration and to fear that methods involving precipitation of the blood proteins might also remove traces of soluble manganese, we eventually substituted for blood a solution having the following composition:

	gm.
Sodium chloride	8.0
Potassium "	0.2
" acid phosphate	0.05
Sodium bicarbonate	1.0
Calcium chloride	0.2

Made up to 1,000 cc. Hydrogen ion concentration = 0.3×10^{-7}.

After shaking up manganese dioxide in this solution the mixture was allowed to stand in an incubator at 38°C. for 6 hours, and then filtered at high pressure. The filter employed consisted of an alundum thimble reenforced by a collodion membrane and fixed in a brass cylinder in such a manner as to be subjected to pressure from an oxygen tank. Filters of this type were shown to permit the

passage quantitatively of soluble manganese salts. The filtrate resulting from the above procedure proved entirely free from manganese, from which fact it seems safe to assume that the manganese dioxide introduced into the mammalian organism, if dissolved by the blood, undergoes this process so slowly as to be negligible.[1]

Finally, we have made *in vitro* agglutination experiments with cat and rabbit serum and manganese dioxide suspensions such as have been used for injection, and after thorough incubation at 38°C. have obtained no evidence that agglutination of particles takes place. It is probable that blood serum could be used to supply protective colloids in making such suspensions instead of acacia.

Quantitative Determination of Manganese and the Normal Manganese Content of Cat Tissues.

We have employed the method for manganese determination developed by Reiman and Minot (5), taking the organs from the animals immediately after they have been bled to death, and using not less than 30 gm. specimens when the latter were obtainable.

The second column of Table I gives the average amount of manganese in mg. per 100 gm. of wet tissue taken from fifteen normal cats. In the third column will be found the average value of the same tissue, except the liver, lung, and spleen, taken from twelve cats 1 hour after an injection of manganese dioxide. With the exception of the kidney the two columns are practically identical, and in the case of this organ the difference is extremely small. It is, of course, necessary to omit the liver, lungs, and spleen from this table, since these organs constitute the chief repository for the manganese dioxide injected.

[1] Recent experiments with manganese dioxide indicate that in the presence of organic matter traces of carbonic acid may cause very fine particles to dissolve. While such solution may be of importance for the final elimination of manganese dioxide, it is of negligible consequence in the 1 hour experiments recorded in this paper.

TABLE I.

Average Manganese Content per 100 Gm. of Wet Tissue in the Organs of Fifteen Normal Cats and of Twelve Cats Injected Intravenously with Manganese Dioxide.

Organ.	Amount of manganese per 100 gm. of wet tissue.	
	In normal animals.	In animals 1 hr. after injection of manganese dioxide.
	mg.	*mg.*
Liver	0.341	
Lung	0.031	
Spleen	0.069	
Kidney	0.136	0.221
Stomach	0.046	0.056
Small intestine	0.044	0.060
Colon	0.086	0.074
Brain	0.035	0.044
Spinal cord	0.059	0.041
Muscle	0.025	0.024
Urine	0	0

Details of an Injection Experiment.

Experiments have been performed upon cats anesthetized with urethane, 2 gm. per kilo, and upon normal animals in which a vein for injection has been exposed under local anesthesia. Blood pressure and respirations have been recorded during injection in many experiments under general anesthesia, and as is indicated by Fig. 1 toxic effects have not been observed. The following protocol illustrates the course of a typical experiment.

Date.—February 4, 1920.
Weight of Cat.—3.7 kilos.
Anesthesia.—37 cc. of 25 per cent urethane solution.
Suspension.—0.14 mg. of manganese per cc., 0.5 per cent sodium chloride, and 0.4 per cent acacia.
Injection.—10 cc. of suspension per kilo.

$$10 \times 3.7 = 37 \text{ cc.}$$
$$37 \times 0.14 = 5.18 \text{ mg. of manganese}$$

Injection Time.—Start, 11.21⅙ a.m.; finish, 11.23½ a.m. Duration 2 minutes, 20 seconds.

Blood samples.			Tracings.	
No.	Time taken.	Length of time after injection.	No.	Time taken.
	a. m.	*min.*		*a. m*
1	11.26½	3	1	11.19
2	11.41½	18	2	11.37
	p. m.			*p. m.*
3	12.26½	63	3	12.20
4	12.46½	83	4	12.40

Rate of Disappearance of Manganese Dioxide from the Circulating Blood.

As a means of indicating the rate at which manganese dioxide particles leave the blood stream, 10 cc. samples of blood were taken at intervals from the carotid artery. As an average procedure four specimens were taken from each cat at varying intervals. Samples of less than 10 cc. would be insufficient to yield positive results if the manganese were still present to the extent of 0.020 mg. per 100 cc. The normal manganese content of cat blood is so low that it is safe to assume that any manganese detectable in a sample of blood not exceeding 25 cc. must be due to the injection. It was found in most cases that taking more than four samples (aggregating 40 cc. of blood) so lowered the blood pressure as to interfere with the circulation. While low blood pressure alone has not altered either the distribution of manganese dioxide or the rate at which it leaves the blood stream, we have not included in these observations experiments in which serious reductions occurred.

Table II displays the data from which certain conclusions may be drawn regarding the rate of elimination. The time interval is indicated by the number of minutes which elapsed between the last drop of the injection and the first drop of blood collected. The actual time required for the process of injection and of blood-taking is insignificant. As an example, the figures in Experiment 5 may be interpreted. The cat weighed 2.4 kilos and was injected with a suspension containing 1.4 mg. of manganese per kilo of cat, or a total of 3.36 mg. of manganese. 3 minutes after the completion of the injection 10 cc. of blood were taken, which upon analysis yielded

0.007 mg. of manganese. The manganese content of the blood is calculated per 100 cc.; therefore in the table the above value appears as 0.070 mg. Again, after an interval of 18 minutes from the time of injection, the blood was found to be negative, which means that 100 cc. contain less than 0.020 mg. of manganese. Examination of Table II indicates that elimination from the circulation is not in any manner dependent upon the dose administered per kilo.

TABLE II.

Rate of Disappearance of Manganese Dioxide from the Circulating Blood in Normal Cats.

Experiment No.	Amount of manganese per kilo of cat.	Total amount of manganese injected.	Amount of manganese per 100 cc. of blood. Length of time after injection.				
			3 min.	8 min.	18 min.	63 min.	68 min.
	mg.	*mg.*					
1	2.45	4.17	0		0		
2	1.40	4.48	0.030		0		
3	1.40	3.22	0.040		0		
4	1.40	3.22	0.046		0		
5	1.40	3.36	0.070		0		
6	2.45	4.17	0.085		0		
7	2.45	6.40	0.093		0		
8	1.40	4.16	0.107		0		
9	0.86	2.75	0.140	0.080	0		
10	1.40	3.92	0.080		0.070	0.040	
11	1.40	5.17	0.138		0.088		
12	2.00	5.20	0.267		0.160		0.088
13	2.00	5.40	0.418		0.244	0.139	

In nine experiments out of the thirteen, the blood is found to be negative after an interval of 18 minutes. In the four in which positive results are found after 18 minutes, though the process of elimination is steady and progressive, the experiment has not been prolonged sufficiently to reach the end-point. Within the limits of injection amounts recorded in Table II elimination is either rapid and complete, or very slow and constant, not depending upon the dose or concentration of the injection but upon some unknown factor in the individual animal.

It has been said that alterations in blood pressure had no effect upon the rate of disappearance of manganese dioxide from the circulating blood. We attempted to alter both distribution and rate of disappearance by a variety of maneuvers but were unsuccessful. Fig. 2 shows an experiment upon a urethanized cat weighing 2.9 kilos in which, after recording the blood pressure, a 25 cc. specimen of blood was taken, and then 35 cc. of a 20 per cent solution of acacia in physiological salt solution were injected. Immediately following this injection 24 cc. of manganese dioxide suspension containing 4.69 mg. of manganese were introduced. The results of analyses upon the blood specimens are given in Table III.

TABLE III.

Disappearance of Manganese Dioxide from the Circulating Blood Following a Large Injection of Acacia Solution.

Amount of manganese per kilo of cat, 1.62 mg. Total amount of manganese injected, 4.69 mg.

Length of time after injection.	Amount of manganese per 100 cc. of blood.
min.	*mg.*
3	0.109
18	0
63	0
83	0
103	0

It is obvious that the high acacia concentration in this animal had no effect in holding particles within the circulating blood. There is complete removal in less than 18 minutes, just as is most often true for normal animals. Analysis of the tissues of this cat showed deposition of manganese dioxide in the organs and in the proportionate amounts characteristic of normal animals, so that neither the existence of acacia in high concentration within the blood vessels nor the very high blood pressure (220 mm. of mercury) in any way affected the result. In another animal under similar conditions of anesthesia the blood pressure was lowered markedly by histamine and remained low during the 1 hour period following manganese dioxide injection. Here again results were obtained which differ in no way from those gained with normal animals.

It is thus shown that two substances, acacia which reduces capillary permeability and histamine which increases it, have no effect either upon the removal from the blood stream or upon the distribution of the particles studied.

When suspensions of manganese dioxide far stronger than those employed in the instances cited in Tables II and III are used, the blood does not become free in 1 hour. In Table IV results are given showing the manganese content of the blood at the end of varying periods of time and following large injections.

TABLE IV.

Manganese Content of the Circulating Blood Following Large Injections.

Length of time after injection.	Amount of manganese injected.	Amount of manganese per 100 cc. of blood.
hrs.	*mg.*	*mg.*
1	40.6	0.480
6	36.1	0.560
18	36.1	0.533
19	36.1	0.245

Distribution of Manganese.

A series of twenty injection experiments was carried out in order to determine the distribution of manganese throughout the body of the cat after an interval ranging from 63 to 83 minutes. An analysis of the liver, lung, spleen, kidney, stomach, small intestine, colon, brain, spinal cord, muscle, blood, and urine of twelve cats gave evidence that the first three organs were the only tissues affected to any significant degree by an intravenous injection of manganese. Table V shows the results obtained with reference to the liver, lung, and spleen.

The weight of manganese normally existing in a particular organ of an injected cat must be deducted from the weight of manganese recovered from it. For example, a liver taken from an injected cat weighs 87 gm.; the manganese content of normal cat liver is 0.341 mg. per 100 gm. of tissue; 87 gm., therefore, contains $\left(\frac{87}{100} \times 0.341\right)$ 0.297 mg. of manganese; the above liver was found to contain 3.740 mg. of manganese; deduct the normal content (3.740 − 0.297) and

the manganese content of this liver resulting from the injection is 3.443 mg. All the values in Table V have been arrived at in this manner.

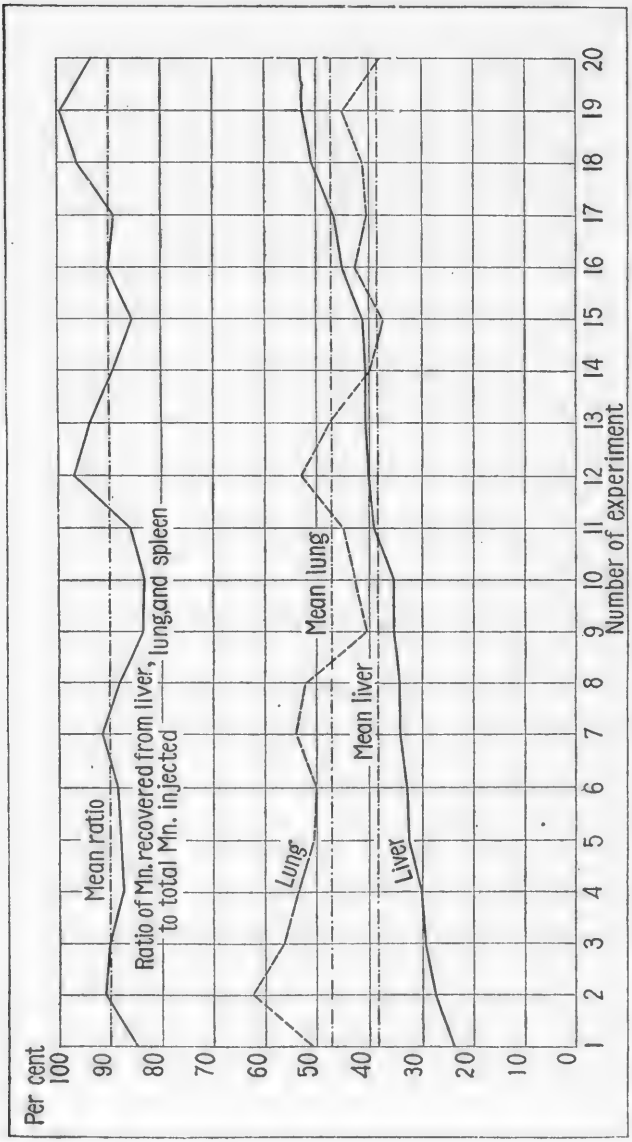
The concentration of the suspension will be seen to vary from 0.100 to 0.245 mg. of manganese per cc. The dose of manganese

TABLE V.

Distribution of Manganese in the Liver, Lung, Spleen, and Other Organs Following the Intravenous Injection of Manganese Dioxide.

Experiment No.	Amount of manganese per cc. of suspension.	Amount of manganese injected.	Per cent of injected manganese in liver, lung, and spleen.	Per cent of injected manganese in other organs.	Per cent of injected manganese in liver.	Per cent of injected manganese in lung.	Per cent of injected manganese in spleen.
	mg.	*mg.*					
1	0.100	4.80	85	15	24	51	10
2	0.165	4.96	92	8	27.5	62.5	2
3	0.245	8.36	90	10	29.3	56.4	4.3
4	0.221	4.20	87.3	12.7	30	53.8	3.5
5	0.245	7.54	88	12	32.7	50.7	4.6
6	0.245	8.07	88.5	11.5	33	50	5.5
7	0.187	4.68	91.4	8.6	34.4	54	3
8	0.245	7.53	88	12	34.5	52	1.5
9	0.245	5.48	83.3	16.7	35.5	40.2	7.6
10	0.245	9.80	83.5	16.5	35.5	42.5	5.5
11	0.245	4.17	86	14	39	45	2
12	0.140	3.92	97	3	40	53	4
13	0.245	7.81	93.7	6.3	40.5	47.5	5.7
14	0.245	6.40	89	11	40.5	40	8.5
15	0.100	7.00	85.5	14.5	41	37.5	7
16	0.245	4.17	90	10	45	42.5	2.5
17	0.245	5.39	89	11	46.5	40.5	2
18	0.245	7.28	96	4	51	41	4
19	0.245	6.20	99	1	52.5	45	1.5
20	0.195	6.05	93	7	53	38	2
Average.....			90	10	38.3	47	4.3

administered varies according to the weight of the cat and the concentration of the suspension. Except in Experiments 1 and 15, 10 cc. of suspension were injected per kilo of cat. In Experiments 1 and 15, 20 cc. per kilo of a comparatively weak suspension were injected. Thus, in Experiment 11 a suspension having a concentration of 0.245 mg. of manganese per cc. was injected into a cat weighing



TEXT-FIG. 1. Percentage concentrations of manganese in liver, lungs, and spleen 1 hour after manganese dioxide injection. For the amounts injected see Table V.

1.7 kilos. If 10 cc. per kilo are injected the dose must contain (0.245 × 17) 4.17 mg. of manganese.

Column 4 gives the per cent of the injected manganese recovered from the liver, lung, and spleen. Column 5 represents the amount of manganese which has escaped the liver, lung, and spleen, and therefore must be deposited in other parts of the body.

Column 6 shows the per cent of the injected manganese recovered from the liver. In Experiment 11, for instance, of the 4.17 mg. of manganese injected into the cat, 1.63 mg. were found in the liver, representing $\left(\frac{1.63}{4.17}\right)$ 39 per cent. Columns 7 and 8 give the same calculations as applied to the lung and spleen respectively. Text-fig. 1 gives graphically the salient facts derived from Table V.

Such variations in the concentration of the suspension as we have employed have had no effect upon the distribution within the organism. It would appear that the work of eliminating the manganese from the blood stream is divided between the liver and the lung in such a manner that they act reciprocally; that is, following injection about 85 per cent of the particles are at once removed by the liver and lung, the lung usually receiving the larger part, but in every instance what is not taken by one of these organs is taken by the other. There is an insignificant amount, 14.7 per cent as an average, in the twenty experiments recorded in Table V, which goes to other organs. The spleen receives a constant slight increment, but the small and variable size of this organ in the cat causes great variation in the amount deposited in it. An average of 4.3 per cent in twenty different spleens represents the activity of this organ. The kidney, intestinal tract, and bone marrow retain manganese dioxide particles in varying and minute amounts, totaling 10 per cent in the experiments recorded in Table V.

DISCUSSION.

The results which we have obtained relative to the rate of disappearance of manganese dioxide from the circulating blood are not paralleled by any other work with injections of non-living material.

Hoffmann and Langerhans (6) employed a suspension of mercuric sulfide containing 0.5 gm. per cc. of water. Large amounts of the suspension were injected. In guinea pigs receiving 3.9 gm. of mercuric sulfide, cells containing cinnibar granules were found in the blood 24 days after injection. It is impossible to determine the history of such particles, and the same is often true in more recent work with carbon, carmine, etc.

With injections of microorganisms the case is different. The experiments of Wyssokowitsch (7), who introduced a variety of yeasts and microorganisms intravenously into dogs and rabbits, demonstrate a partial or complete disappearance of these particles from the circulating blood shortly after injection. In two experiments on yeasts a few spores were left at the end of 1 hour, though the injection was enormous. Saprophytic bacteria such as *B. subtilis* disappeared with extreme rapidity and completeness. In one instance when 1 cc. of a concentrated suspension was introduced in the rabbit the blood proved sterile after 15 minutes. With organisms such as *B. anthracis* and *Staphylococcus aureus* the initial rates of removal from the blood stream seem comparable to those obtained with saprophytic organisms, but in these instances there is later a tendency for the appearance in the blood of large numbers of organisms, and this phenomenon is followed by the death of the animal. Wyssokowitsch (7) had no conception of many of the reactions which may be aroused by injected protein material. His results merely indicate the promptitude with which the blood gets rid of very large numbers of living foreign particles. Bull (8) has examined more fully the rate and manner of disappearance of streptococci, pneumococci, and typhoid bacilli from the blood of the rabbit. The manner in which this animal meets such infection varies with the aggressive power of the bacteria employed and the defensive power of the animal, but essentially removal from the blood stream seems to follow the lines already laid down by Wyssokowitsch (7). Thus, with streptococci of low virulence Bull (8) reports an injection into the ear vein of 0.25 cc. per kilo of a bouillon culture of streptococci. After 30 minutes 52 colonies were obtained, following the use of a uniform technique for blood culture. After 2 hours 1 colony was found, after 48 hours 100 colonies were obtained, and after 72 hours the colonies became so numerous as to be uncountable. Later another rabbit injected with the same strain and dose of streptococci, attenuated in virulence as a result of continued cultivation on artificial media, showed sterility of the blood at the end of 2 hours, and this sterility was maintained until 168 hours had passed, when there was a reappearance of streptococci in the blood stream followed shortly by the death of the animal.

With typhoid bacilli Bull (8) presents the following experiment.[2]

"A rabbit weighing 2,000 gm. was given $\frac{1}{10}$ of a 24 hour agar slant of typhoid bacilli in the ear vein. Blood cultures made from the heart at stated intervals gave the results indicated in Table I."

[2] Bull, C. G., *J. Exp. Med.*, 1915, xxii, 476.

TABLE I.

Time after injection.	No. of colonies per cc.
min.	
1	10,000,000
2	2,500,000
5	100,000
15	40
20	1

Although we cannot make a direct and final comparison between intravenous bacterial injections and intravenous injections of non-living material such as the manganese dioxide employed in the present study, it seems established that in as far as the immediate removal from the blood stream is concerned the two types of injection are treated similarly by the animal. Furthermore, although the cat differs from other animals in the organs which take foreign material from the blood stream, it does not seem to differ in the rate at which material is removed.

Bull (8), in the case of pneumococci and typhoid bacilli, has related the speed and thoroughness of blood sterilization in the intravenously injected rabbit to an *in vivo* agglutination of the organisms in question by means of immune bodies present in the animal or introduced just prior to injection of the culture employed. He, however, does not make explicit just how this agglutination brings about blood sterilization except in instances in which the blood infection is massive and there is evident blockage of the vessels, particularly in the lungs, by large clumps of organisms. When, however, the bacteria are less numerous, their clumping is not mentioned as an important factor in removal from the blood stream but seems to signal a condition which is followed promptly by phagocytosis. Finally, it is of importance to note that the organs in which the clumped bacteria are chiefly removed are designated by Bull (8) as liver, spleen, and lungs. These are the points at which non-living and non-agglutinated living particles accumulate. If, in Bull's (8) observations, embolism offered an explanation for removal of the organisms from the circulating blood, there is no obvious reason why the capillaries of other organs such as muscle should not take part in the sterilization. That they do not is thus evidence for some specific type of activity in certain organs resulting in bacterial removal from the blood and favored in some way, not understood, by the action of immune bodies in bringing about agglutination.

Kitagawa (9) injected pneumococci unagglutinable by the serum of the rabbit *in vitro* and unagglutinable by the blood of the same animal *in vivo*. The latter fact he proved by isolating samples of infected blood between ligatures placed about the larger blood vessels. He concluded that the disappearance of the

organisms from the blood stream "could not be due to a purely mechanical filtration of agglutinated masses by the tissue capillaries, but that it necessitated some active biological cooperation of the capillary cells" (10).[3]

In the present work the failure to affect the rate of removal of manganese dioxide from the circulating blood and to alter the ratio of distribution in different organs through changes in blood pressure and in concentration of the suspensions employed, points again to the fact that some specific activity on the part of particular cells in certain organs is responsible for removal and that simple mechanical factors are of little or no importance.

Mallory (11) has called attention to the fact that practically all vascular endothelium can become phagocytic. Thus, if blood vessels are injured it can be shown that during the process of wound repair phagocytic cells arise from the proliferating endothelium (12). There has long been a controversy as to whether such cells did not wander into the traumatized area from recognized sites of origin as the milk patches of the omentum. Foot (13) in recent experiments upon this subject found that proliferating vascular endothelium is markedly phagocytic. He discusses the question as to whether connective tissue cells in foci of inflammation may not also contribute phagocytes, but concludes that they do not.

It therefore becomes apparent that phagocytosis is a potential property of vascular endothelium. In certain localities, such as in the capillaries of muscle, this power does not seem to be exhibited except following injury and resulting proliferation. Other regions, notably the liver, spleen, and lung of the cat, possess vascular endothelium which under normal conditions is capable of removing foreign particulate material from the circulating blood. With bacteria there is evidence that the ability of the endothelium to perform this function varies. Manwaring and Coe (10) perfused the livers of normal and immunized rabbits with defibrinated blood to which pneumococci had been added. In the normal rabbit even a dozen passages through the liver reduce the pneumococcic count but slightly, whereas in the animal actively immunized against pneumococci two to five passages sterilize the perfusing blood. This effect is attributed to an opsonin which so alters the pneumococci as to cause them to adhere to the endothelial cells lining the liver capillaries. The same opsonin is practically inoperative in promoting bacterial removal by endothelial cells of extrahepatic tissues.

Hopkins and Parker (14) injected streptococci intravenously in cats and rabbits and found an initial deposition for the cat in the lungs, liver, and spleen with slight lodgment in bone marrow, lymph nodes, muscle, and kidney. The lung of the cat received the largest number of organisms, whereas in the rabbit the

[3] Kitagawa, K. J., quoted by Manwaring and Coe (10), p. 402.

lung is far less active than the liver. In a subsequent paper Lund, Shaw, and Drinker (15) bring out the same difference between the cat and the rabbit in the handling of manganese dioxide particles. These animals, therefore, exhibit an initial capacity to remove foreign material from the circulating blood which is the same for living and non-living material provided the particles in question are of approximately the same size. Hopkins and Parker (14) report that in the rabbit which has been partially immunized against streptococci the lung exhibits an initial power to remove the organisms equal to that seen in normal cats. Here, again, there is evidence of varying activity which in the last analysis seems to reside in the capillary endothelium. While in the case of carbon and starch (16, 17) it has been shown that there are non-specific opsonins which promote phagocytosis, it has never been possible to display variation in the deposition or rate of removal of non-protein material as a result of repeated injections. While, therefore, one may conclude that the initial reaction of the animal against foreign particles is the same, in the case of bacteria subsequent reactions accomplishing removal from the blood stream may be modified as a result of the appearance of immune bodies.

The actual mechanism by which the particles are removed is evidently highly specialized in certain organs. It involves, first, an arrest of the particles as they pass through the capillaries. There is no evidence in our own histological preparations that this arrest is the result of embolism, nor, as has been pointed out, is there evidence in experiments with bacteria that embolism plays an important part. In the case of the lung Hopkins and Parker (14) make the following statement.[4]

"In lung sections of animals killed 10 to 30 minutes after injection it is clear that some of the cocci are held within the leucocytes in the capillaries. It is equally clear that the majority are not located within leucocytes. Many of them lie in large mononuclear cells which are probably the swollen endothelium of the capillaries; others lie in an eosin-staining matrix which may represent a section through endothelial protoplasm, or possibly some substance from the blood deposited about them.

"In order to study this point more accurately, we have etherized and killed cats within 30 minutes after injection, washed the lungs as free as possible by perfusion with salt solution, and then fixed them by injecting Helly's fluid under moderate pressure in order to keep the capillaries distended. In sections fixed in this way the streptococci are not washed from the capillaries; even leucocytes are still present in moderate numbers. Of the mechanism by which the cocci are held, however, we are still in doubt, though many appear to be in endothelial cells."

Foot (13), who made large injections of a carbon suspension in rabbits daily over a considerable period, states that the capillary endothelial cells take up the particles. Slavjansky (18), several days after a heavy intravenous injection of mercuric sulfide in rabbits, found cells containing the substance free in the alveoli.

[4] Hopkins and Parker (14), p. 11.

He believed, as did Tchistovitch (19), on the basis of similar observations, that these cells were derived from the macrophages of the circulating blood. In all the instances cited the injections were extremely large. Sewell (20) injected a rabbit intravenously with a carbon suspension, the strength of which is not indicated. He, however, filtered it carefully and seems to have avoided the possibility of pulmonary embolism, a contingency from which Foot's (13) experiments would not seem to be free. He found little or no carbon deposited in the rabbit lung and no carbon-containing cells in the alveoli, and contends that certain of the alveolar cells have the property of taking up particles from the air but not from the alveolar structure upon which they rest.

In the present study histological preparations made from cats killed 1 hour after injection of manganese dioxide have shown the material in the following locations in the lung. (1) A very small amount, seen only after protracted search, is still within the capillaries and apparently free. These granules are usually single or in small groups. They do not seem to be attached to any cells, but evidently are not progressing through the lungs, since we have found them so situated when the circulating blood was entirely free from manganese. (2) Particles are found in or on the surface of the capillary endothelial cells. This fraction represents the larger part of the lung content. (3) Groups of particles, not agglutinated, lie within the alveolar septa, particularly at points where septal junctions occur. These points represent the second most important site of the particles. (4) Granules are seen within large lining alveolar cells which occur particularly at points of alveolar junction. Occasionally these cells lie free within the alveoli.

We believe that the initial lodgment of practically all particles in the lung is within or upon the lining capillary endothelium. This deposition of manganese dioxide is very uniform through the different lobes of the lung. As this deposition must express the extent of the capillary bed at the moment of injection, it is a fair assumption that the capillary blood flow is proportionally uniform through the different lung lobes moment by moment.

The function of the liver in removing foreign particles from the blood stream is of greater significance than that of the lung. In the cat we have found a large initial deposition of manganese dioxide in the lungs, but we have been able to show[5] that from the 3rd

[5] Drinker, C. K., and Shaw, L. A., unpublished observations.

hour following injection the material in the lungs begins to diminish, accumulating at first in the liver and from this organ passing into the intestinal canal. Furthermore, as pointed out by Lund, Shaw, and Drinker (15), the lungs of the common laboratory animals, with the exception of the cat, achieve only a slight share of blood sterilization. The capillary bed of the liver differs from that of the lung in that the lining endothelium contains elements, the stellate cells of Kupffer (21), which have long been known to be actively phagocytic. It has also been contended that these capillary walls are incomplete (22), permitting material in the portal system to move directly into the liver cells. Whether or not this is true, it seems certain that the liver cells themselves have little to do with the initial removal of foreign material from the portal blood.

The initial positions of manganese dioxide in the livers of cats injected as in these experiments are as follows: (1) Particles are found free within the capillaries. Just as with the lung, even when the circulating blood of the animal shows no manganese, occasional single particles and groups of particles are found in the capillaries. They are not intracellular and as far as can be seen are completely unattached to the capillary walls. Shipley and Cunningham (23) immersed the omentum of the decerebrate cat in an India ink suspension, and after an interval found many fine, free carbon particles in the liver capillaries. They present evidence that this material is taken up by the omental capillaries and express surprise upon finding it outside cells within the blood channels of the liver. Apparently the particles which we introduced directly into the blood stream became arrested in the same way, but none of the sections has given any evidence of capillary blockage. (2) Large collections of particles are seen in or upon the Kupffer cells. These cells unquestionably account for the great activity of the liver in clearing the circulation of foreign material. The ultimate fate of substances engulfed by them is unknown. There is no evidence which indicates that material once deposited in the liver becomes free in the sense of passing back into the circulation to be deposited elsewhere. Once taken up by the liver, particles may be considered to be on the way toward removal from the body. (3) Occasional granules are found upon the surface of the liver cells. At the end of 1 hour these are very few and repre-

sent an unimportant method of disposal, a point against the theory of the immediate relation of the liver parenchyma to the portal blood.

In the case of the spleen manganese dioxide is found diffusely scattered through the pulp. Many of the particles are free and many have been taken up by large phagocytic cells. As with other types of injection, the Malpighian corpuscles are quite free from particles. The capillary circulation of the spleen is open, and diffuse deposition of material through the pulp is the natural expectation under such mechanical conditions. The presence of phagocytic endothelial cells throughout the pulp assures the seizure of material which drifts into this tissue, but the ultimate fate of particles so phagocytosed is unknown.

In addition to the lungs, liver, and spleen, we have found varying small deposits of manganese dioxide in other organs. The bone marrow remains comparatively free. Bacterial lodgment often occurs in the marrow, which is known to be a reservoir of endothelial phagocytes. In spite of these facts we have found it of little or no importance in clearing the blood of manganese dioxide. The kidney and the intestinal tract in general take the material in varying small amounts in different animals. Often they have remained absolutely negative and at best are of little importance. Muscle, skin, and nervous tissue are invariably free. In this relation it may be noted that while Hopkins and Parker (14) in their studies of streptococcic lodgment found minimal numbers initially removed in muscle, they attribute the eventual serious consequences in their animals to proliferation of these muscle organisms, finding that those removed by the liver, lungs, and spleen have far less chance for growth.

CONCLUSIONS.

1. Manganese dioxide suspended in an acacia-sodium chloride solution provides a non-toxic injection which in the present experiments has contained no particles larger than 1μ and which, when deposited in the body, can be determined quantitatively and seen microscopically.

2. Intravenous injections have been made under precautions which preclude removal from the blood or deposition in organs through simple capillary blockage.

3. In nine experiments out of thirteen the circulating blood contained no manganese after 18 minutes. In the four remaining instances there was a steady slight elimination which was incomplete at the end of 1 hour. Within certain limits the rate of removal from the circulating blood and the sites of deposition in the animal are not influenced by the concentration of the suspension, the blood pressure, or antecedent introduction of acacia or histamine.

4. In the cat amounts of manganese dioxide varying between 9.8 and 3.9 mg. of manganese and containing from 50,000,000,000 to 10,000,000,000 particles, if injected intravenously, permit recovery at the end of 1 hour of 90 per cent of the material in the lungs, liver, and spleen in the following proportions: lungs 47 per cent; liver 38.3 per cent; spleen 4.3 per cent.

5. These experiments, coupled with correlative results by other investigators, make it clear that in certain organs—the lungs, liver, and spleen of the cat—the vascular endothelium possesses phagocytic power rendering the capillaries permeable to particulate material as well as to gases, liquids, and dissolved substances.

BIBLIOGRAPHY.

1. Bouffard, G., *Ann. Inst. Pasteur*, 1906, xx, 539.
2. Goldmann, E. E., *Beitr. klin. Chir.*, 1909, lxiv, 192; 1912, lxxviii, 1.
3. Ponfick, E., *Virchows Arch. path. Anat.*, 1869, xlviii, 1.
4. Voigt, J., *Biochem. Z.*, 1914, lxii, 280; lxiii, 409; 1915, lxviii, 477; 1918, lxxxix, 220.
5. Reiman, C. K., and Minot, A. S., *J. Biol. Chem.*, 1920, xlii, 329.
6. Hoffmann, F. A., and Langerhans, P., *Virchows Arch. path. Anat.*, 1869, xlviii, 303.
7. Wyssokowitsch, W., *Z. Hyg. u. Infectionskrankh.*, 1886, i, 3.
8. Bull, C. G., *J. Exp. Med.*, 1914, xx, 237; 1915, xxii, 457, 466, 475, 484.
9. Kitagawa, K. J., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 213.
10. Manwaring, W. H., and Coe, H. C., *J. Immunol.*, 1916, i, 401.
11. Mallory, F. B., *J. Exp. Med.*, 1898, iii, 611. McJunkin, F. A., *Am. J. Anat.*, 1919, xxv, 27. A useful summary of the literature upon this subject has been published by Evans, F. A., *Studies from the Department of Pathology of the College of Physicians and Surgeons, Columbia University*, 1917, xvi, No. 21.
12. Evans, H. M., *Am. J. Physiol.*, 1915, xxxvii, 243.
13. Foot, N. C., *J. Med. Research*, 1919, xl, 353.
14. Hopkins, J. G., and Parker, J. T., *J. Exp. Med.*, 1918, xxvii, 1.

15. Lund, C. C., Shaw, L. A., and Drinker, C. K., *J. Exp. Med.*, 1921, xxxiii, (in press).
16. Porges, O., *Z. Immunitätsforsch., Orig.*, 1909, ii, 4.
17. Ouweleen, J., *Arch. ges. Physiol.*, 1916, clxiv, 457; 1917, clxvi, 88; clxviii, 372.
18. Slavjansky, K., *Virchows Arch. path. Anat.*, 1869, xlviii, 326.
19. Tchistovitch, N., *Ann. Inst. Pasteur*, 1889, iii, 337.
20. Sewell, W. T., *J. Path. and Bact.*, 1918–19, xxii, 40.
21. Kupffer, C. W., *Arch. mikr. Anat.*, 1899, liv, 254.
22. Schäfer, E. A., *Proc. Roy. Soc. Edinburgh*, 1901–03, xxiv, pt. 1, 65. Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1906, lxxviii, 455.
23. Shipley, P. G., and Cunningham, R. S., *Am. J. Physiol.*, 1916, xl, 75.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Injection of manganese dioxide suspension into the femoral vein. Upper curve respiration, downstrokes representing inspiration. Lower curve blood pressure. Base-line 1 minute intervals marking upward; injection signalled downward. Between Tracings 1, 2, 3, and 4, specimens of blood were withdrawn for analysis.

PLATE 4.

FIG. 2. Tracing 1 indicates normal blood pressure. Between this tracing and No. 2, 25 cc. of blood were withdrawn from the carotid artery. Tracing 2 indicates first the low blood pressure consequent upon this hemorrhage and then the rise attendant upon acacia solution and manganese dioxide solution injections. Tracings 3, 4, and 5 indicate the blood pressure during the remainder of the experiment. The gradual fall of pressure is due to withdrawal of blood for analysis.

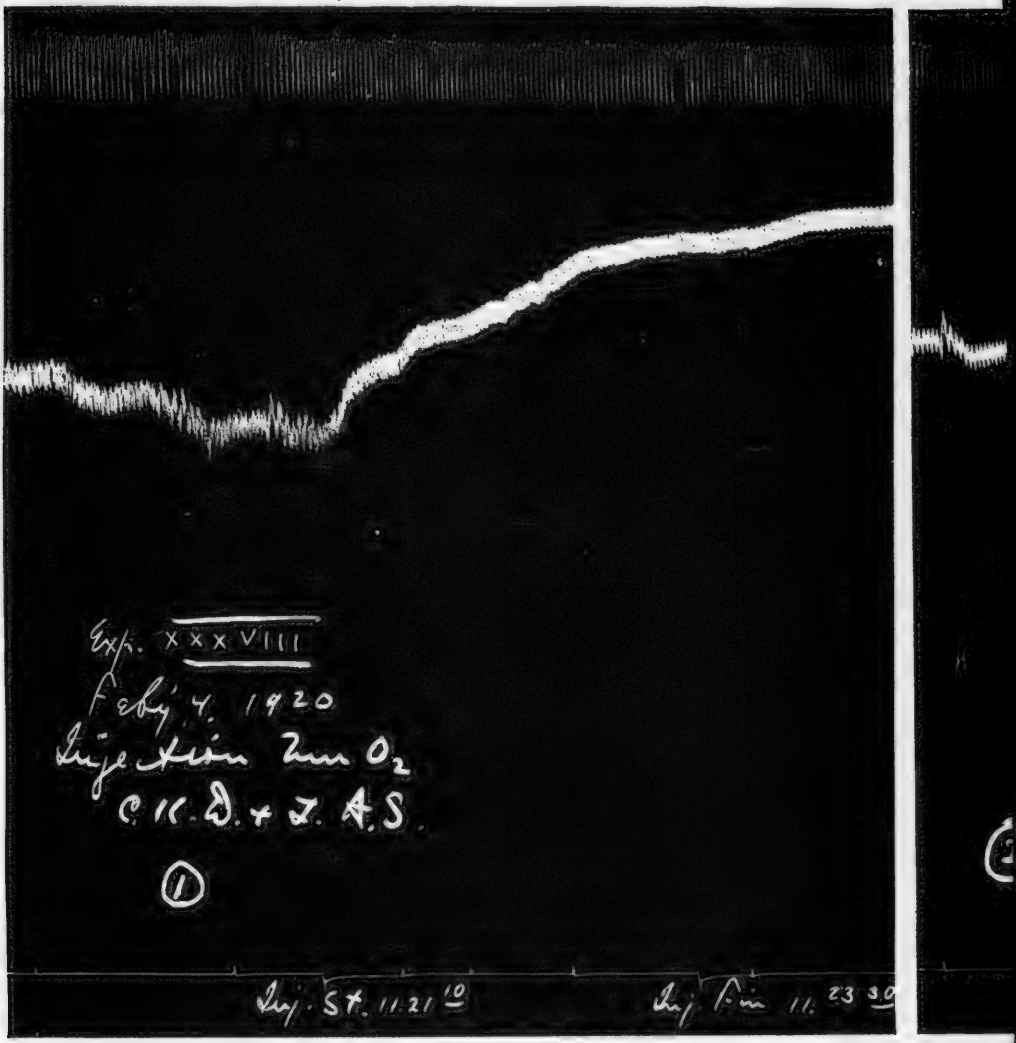

Exp. XXXVIII
Feby 4. 1920
Injection 2cm O_2
C.H.D. + I.A.S.
(1)
Inj. St. 11.21 10
Inj. Fin 11. 23 30

FIG. 1.

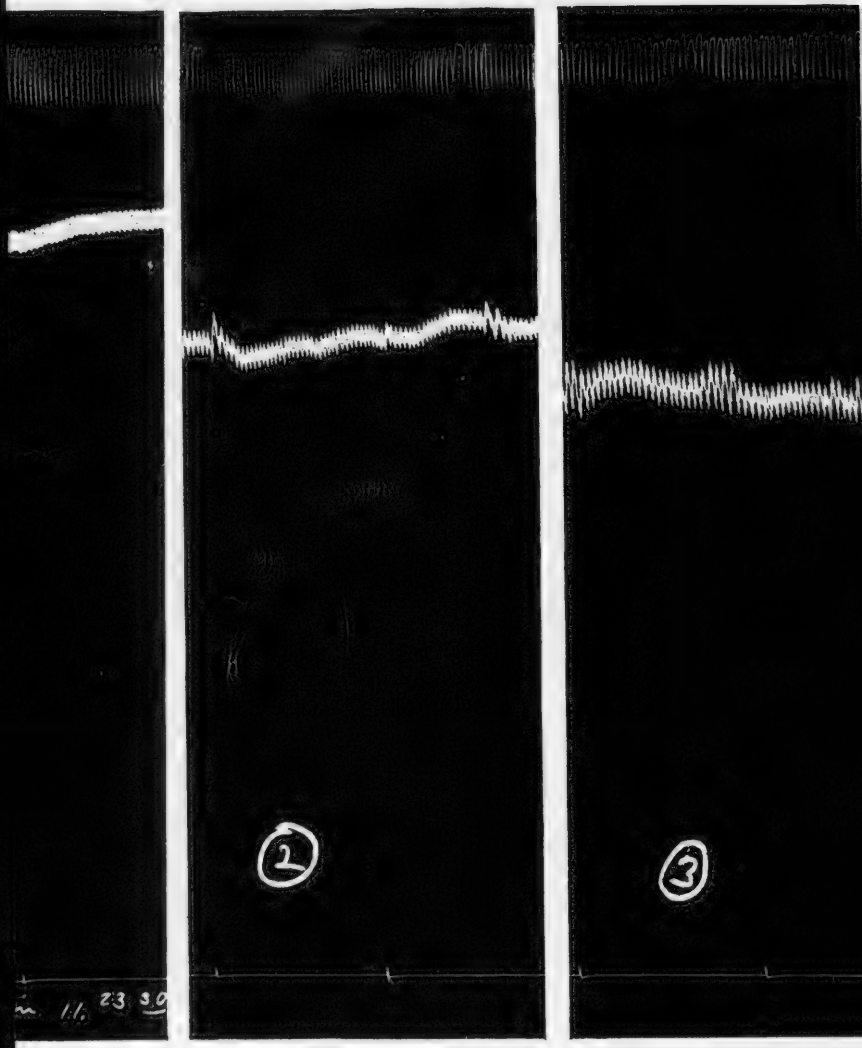
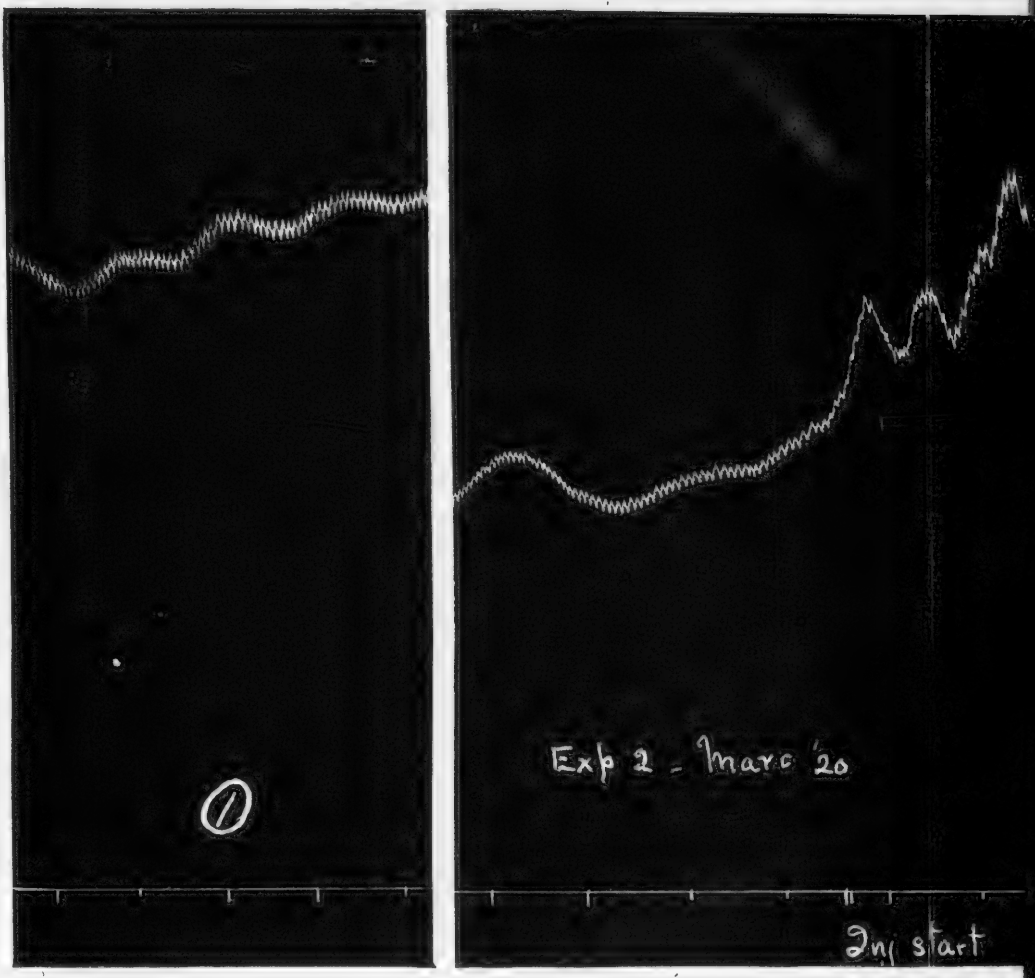


FIG. 1.

PLATE 3.

ed intravenously.)


0
Exp 2 - March 20
Inj start

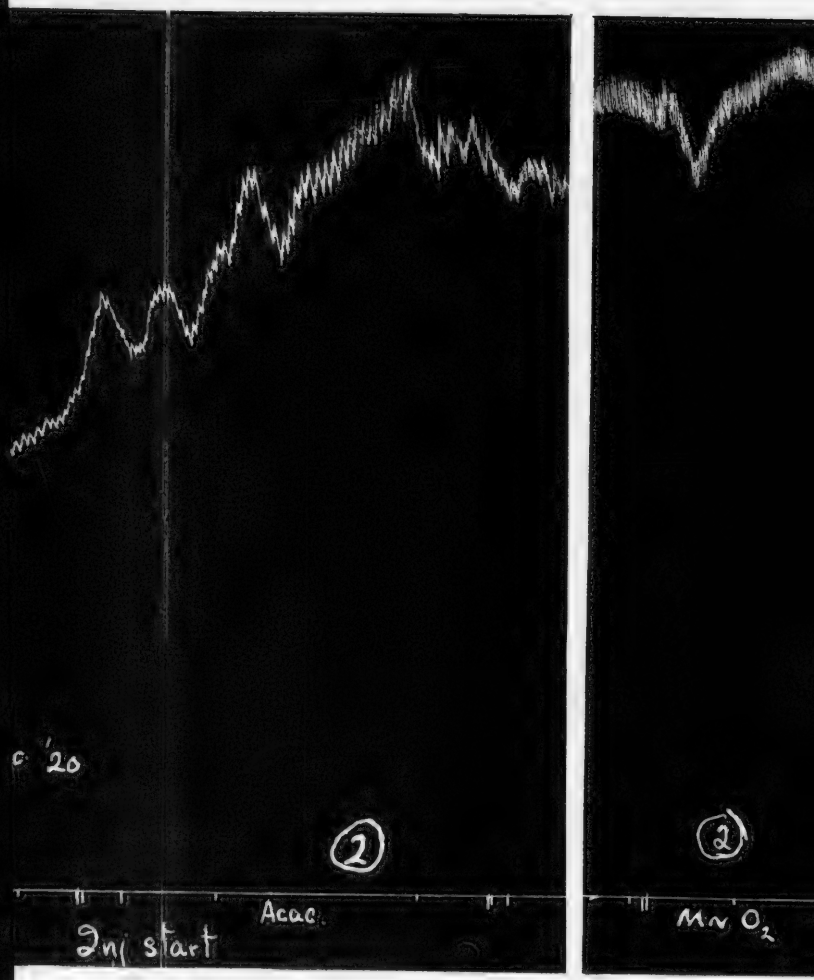


FIG. 2.

PLATE 4.

(Drinker and Shaw: Manganese dioxide administered intravenously.)

STUDY OF THE CLASSIFICATION OF MENINGOCOCCI.*

By AUGUSTUS B. WADSWORTH, M.D., RUTH GILBERT, AND ALICE HUTTON.

(*From the Division of Laboratories and Research of the New York State Department of Health, Albany.*)

(Received for publication, July 9, 1920.)

Work on the classification of meningococci was started in January, 1918, in order to determine whether it was possible to classify by their agglutination and antigenic properties a sufficient number of strains among the cultures at our disposal, so that satisfactory representative cultures could be selected for standardizing serums or immunizing horses. At that time 53 meningococcus cultures were being used in these laboratories in immunizing horses for the production of antimeningococcus serum and for the standardization of the serum by agglutination tests. By the methods of immunization then in use no attention was paid to serological differences, except that the strains of meningococci which showed the least agglutination in the serum of the horses under immunization were injected more frequently than the others. The objection to this procedure is evident, for antibodies may be produced for the strains which show very little agglutination, to the detriment of antibody production for the strains which are more agglutinable. It was thought that a better balance might be obtained by injecting representative cultures in equal amounts, if it was found that a few cultures representative of all or nearly all meningococci could be selected.

The principal methods used to differentiate meningococci into groups have been the agglutination test and the absorption of agglutinin test. Complement fixation and virulence tests have been of questionable value owing to difficulty of technique and to the slight or variable pathogenicity of the meningococcus for small laboratory animals.

* Presented at the 47th annual meeting of the American Public Health Association, New Orleans, La., October 27 to 30, 1919.

Technique for the Agglutination Tests.—The meningococcus stock cultures were kept on serum dextrose agar or semisolid ascitic fluid agar. Transfers for agglutination tests were made on dextrose serum agar slants and incubated at 37°C. for 18 hours. Suspensions were made up with 0.85 per cent saline solution to a density equal to that of barium sulfate Standard No. 3, which approximates 2,000 million cocci per cc. The serum dilutions were made up with 0.85 per cent saline solution. The agglutination tests were set up in small racks in 3 by $\frac{3}{8}$ inch tubes, equal parts (0.2 cc. each) of serum dilution and culture suspension being used for each tube. Incubation was at 55°C. for 24 hours. The tubes were shaken 3 hours before reading, as it had been found that shaking increased and made more uniform the agglutination titer of the serum.

In some of the tests the cultures were grown on plain dextrose agar for 18 hours at 37°C., the growth was suspended in 0.85 per cent saline solution, the suspensions were heated at 65°C. for 30 minutes, and 0.5 per cent phenol was added. They were then diluted to the required density.

Technique for the Absorption Tests.—For the absorption tests a method, based largely upon the observations of Tulloch[1] and of Gordon,[2] was devised and tested by suspending in a serum its homologous culture under different conditions. Two densities of culture suspension, both heated and unheated, and two temperatures of incubation were used. The results are shown in Table I. Satisfactory absorption of the agglutinins took place only when the heated, dense suspensions of culture, incubated at 55°C., were used.

A dense suspension containing about 30,000 million cocci per cc. was secured by suspending in 0.85 per cent saline solution the 18 hour growth on dextrose agar, which had been incubated at 37°C., then heating to 65°C. for $\frac{1}{2}$ hour, and adding 0.5 per cent phenol.

[1] Tulloch, W. J., On the differentiation by means of the absorption of agglutinin test for types of meningococci (etc.), *J. Roy. Army Med. Corps*, 1917, xxix, 66.

[2] Gordon, M. H., Bacteriological studies in the pathology and preventive control of cerebro-spinal fever among the forces during 1915 and 1916. I.—The definition of the meningococcus, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

Equal parts of the serum, diluted to 1:25 with saline solution, and the suspension of cocci to be tested were thoroughly mixed and incubated at 55°C. for 2 hours and then placed in the ice box at 5–8°C. The next morning the tubes were centrifugalized at high speed for ½ hour, or longer if necessary, to throw down all the bacteria, and the supernatant fluid was decanted and tested against the homologous culture of the serum. A sample of the same serum was incubated, centrifugalized, and tested in the same dilutions as the saturated serum.

TABLE I.

Degrees of Absorption with Heated and Unheated Suspension, Incubated at 37° and 55°C., and a Thin and a Dense Suspension.

A Type II serum was saturated with its homologous culture under varying conditions, and after incubation and centrifugalization the supernatant fluid was decanted and tested in a series of dilutions against its homologous culture according to the standard method.

Suspension.	No. of cocci per cc.	Temperature at which culture was heated for ½ hr.	Temperature at which culture was incubated with serum for 2 hrs.	Agglutination of supernatant fluid with homologous culture, Type II.
	millions	°C.	°C.	
A	5,000	65	55	1:400
AA	5,000	65	37	1:400
B	30,000	65	55	0
BB	30,000	65	37	1:100
C	5,000		55	1:600
CC	5,000		37	1:600
D	30,000		55	1:300
DD	30,000		37	1:600

The monovalent type serums were produced in rabbits according to the method suggested by Amoss.[3]

RESULTS.

Standard suspensions and serums of the four types of Gordon were sent to this laboratory by the British Medical Research Committee. Living cultures of the four types of meningococci were sent

[3] Amoss, H. L., quoted by Flexner, S., Mode of infection, means of prevention and specific treatment of epidemic meningitis, *J. Am. Med. Assn.*, 1917, lxix, 639.

to us by Dr. W. H. Park, of the Department of Health of the City of New York, who had received them from Colonel Gordon. Tests were made of the standard suspensions with the standard serums and with our monovalent normal, para, and irregular serums; also the standard serums were tested with our stock cultures. The results of the tests were not altogether clear-cut and satisfactory because the standard serums and suspensions were not tested until after the expiration date recorded on them. It was very clearly indicated, however, that Types I, II, and III of Gordon corresponded respectively to the normal, para, and irregular types. The same relation was indicated by a study of the living cultures of Types I, II, and III of Gordon.

We were unable to designate any of the cultures of our series as belonging to Type IV of Gordon. The Type IV standard suspension showed only a trace of agglutination in the homologous serum, but when tested with the normal, para, and irregular serums, it was agglutinated in a 1:100 dilution by the para type serum, and in a 1:300 dilution by the irregular type serum. These reactions, together with those of the Type IV living culture from Dr. Park, seem to indicate that Type IV may not be a separate group but possibly a poorly agglutinating strain belonging to Type II or III.

Agglutination tests were made with 53 stock cultures in the monovalent rabbit serums produced against the selected strains of normal, para, and irregular meningococci. 43 of the 53 cultures were classified into three distinct groups: six as Type I, thirteen as Type II, and twenty-four as Type III. One culture was agglutinated in Serums I and III, two groups which are said to be related. Nine cultures could not be classified into these groups because they did not agglutinate in sufficiently high dilutions of the four monovalent type serums which were used to classify the other cultures. The type serums employed comprised one of Type I, two of Type II, and one of Type III. Some of the unclassified cultures might conceivably be cultures which were not readily agglutinated. Others might only have failed to react with the particular monovalent serum with which they were tested. With monovalent serums produced with other representatives of the groups they might have been agglutinated.

The three types, Nos. I, II, and III, the recognition of which was based upon the agglutination test, seemed to be distinct when the absorption test was used, in that Type I culture absorbed the specific agglutinins from Type I serum, and did not absorb them from Type II or Type III serum; likewise Type II and Type III cultures absorbed the agglutinins from their respective homologous serums and not from the other types.

Stability of the Serological Types of Meningococci.

The constancy of the reactions of meningococcus strains has at times been questioned; that is, whether a culture at one time gives reactions that would indicate its classification in one group and at a later time its classification in another group. The reactions with at least some strains may be constant for considerable periods of time, for tests at weekly intervals with six strains under cultivation on the standard media used for all strains for 2 years have shown no change so far as the agglutination reaction is concerned.

Variations in Reaction of Four Type II Cultures and One Type IV.

A study was made of the variations in reaction among the cultures of our series. We record here the differences found in some of the Type II meningococcus cultures, and in the culture which we received as a Gordon Type IV.

Tulloch[4] and others have noted that the strains of Type II differ in their agglutination reactions, and Dopter and Pauron[5] suggest that their group of parameningococci which corresponds to the Type II of Gordon might be divided into three subgroups. Both French and British workers have noted the relation between Types II and IV of Gordon.

Monovalent serums obtained from rabbits immunized against Type II Cultures 1 and 4 agglutinated Type II Cultures 1, 4, 46, and G2 (the Gordon Type II culture received from Dr. Park). Monovalent

[4] Tulloch, W. J., Agglutination and absorption of agglutinin reaction, *J. Roy. Army Med. Corps*, 1918, xxx, 115.

[5] Dopter and Pauron, Différenciation des paraméningocoques entre eux par la saturation des agglutinins, *Compt. rend. Soc. biol.*, 1914, lxxvii, 231.

serums obtained by immunization with Type II Cultures 46 and G2 agglutinated in high dilution Type II Cultures 46 and G2, but did not agglutinate Type II Cultures 1 and 4 except in low dilution.

Type II Cultures 46 and G2 absorbed the agglutinins from serum obtained by immunization with Type II Culture 4, and these cultures also absorbed the Type II agglutinins from serum obtained by immunization with Cultures 46 and G2. It is evident that the agglutination reaction alone failed to bring out the relation of these cultures, but that in absorption tests they reacted similarly. It is nevertheless evident from the agglutination reaction that Cultures 1 and 4 might be said to represent one subgroup and Cultures 46 and G2 another.

No cultures were secured which typified Gordon's Type IV save the culture which came originally from Gordon and which we designated as G4. A monovalent serum obtained from a rabbit immunized with this culture agglutinated in high dilution Type II Cultures 1, 4, 46, and G2, but did not agglutinate its homologous culture, No. G4, except in low dilution. It is, therefore, a culture which is not readily agglutinated but seems to be active as an antigen. Culture G4 did not absorb the agglutinins for Type II cultures from the serum of a rabbit immunized with Type II Culture 4, but absorbed these agglutinins from serums obtained by immunization with Nos. 46 and G2. Although not readily agglutinable, the culture was apparently active as an antigen in the development of Type II agglutinins, and was capable of absorbing agglutinins for some, but not all Type II cultures from the homologous serum. Although a relation between Culture G4 and the second group of Gordon was thus indicated, it was the only culture of our series which was sufficiently definite in its reactions to suggest the fourth group of Gordon.[6]

It seems possible that Type II is a complex group and that its members cannot be properly differentiated by the agglutination reaction, although strains which react typically may be classified in it.

These irregular reactions of agglutination and absorption suggested that the agglutination reaction is not a satisfactory basis for a final

[6] Further study is now being made of the variations in agglutination and absorption of the cultures which have been reported to comprise the fourth group of Gordon.

classification, although it might be of value in differentiating groups so that representative strains could be selected for certain purposes such as the immunization of animals to obtain polyvalent serums. That the selection of the representative strains based upon the results of this study was of value in producing a polyvalent serum in horses is well shown by the fact that such serum agglutinated all cultures of our series in high dilution.

CONCLUSION.

The meningococcus, like some other pathogenic species, varies in its agglutination in immune serum, some strains being readily agglutinable while others agglutinate with difficulty in their homologous serum as well as in heterologous serums. The different strains appear to vary also in their action as antigens. In order to secure representative strains, therefore, it was thought necessary to consider the antigenic action as well as the agglutinability of the cultures.

COMPARISON OF THE POTENCY OF POLYVALENT ANTIMENINGOCOCCUS SERUM PRODUCED WITH FOUR AND SIX REPRESENTATIVE STRAINS AND THAT PRODUCED WITH SIXTY STRAINS, AS DETERMINED BY THE AGGLUTINATION TITER.*

By AUGUSTUS B. WADSWORTH, M.D.

(*From the Division of Laboratories and Research of the New York State Department of Health, Albany.*)

(Received for publication, July 9, 1920.)

In the treatment of cerebrospinal fever by antimeningococcus serum the polyvalency of the serum has been considered of the utmost importance. Yet the potency of the polyvalent serum against the individual strain may be sacrificed by immunizing with a large number of strains. The technical difficulties of developing such a serum make its production onerous, while the methods of testing its therapeutic potency are also burdensome and far from satisfactory. Furthermore, in New York State the regulations for the production and standardization of antimeningococcus serum for sale require immunization with and tests against only four representative strains. Manufacturers then might not use more than the four strains required and it is important to determine what effect this procedure would have on the polyvalency of the serum.

Although the agglutination test is not a satisfactory method of determining therapeutic potency, it is the only one that suggests the degree of polyvalency. The practical value of complement fixation or of measuring the bacteriotropin content has not been demonstrated, and there is no satisfactory protection test.

In this study one horse was immunized with four strains, another with six, and the serums obtained from these horses were compared

* Presented at the 47th annual meeting of the American Public Health Association, New Orleans, La., October 27 to 30, 1919.

with the serum obtained from a horse which had been immunized with the regular stock meningococcus cultures—60 strains in all, including the six which were used in the immunization of the horses under investigation. The serum from the horse immunized with the large number of cultures was representative of the classical method, since it had been found to compare favorably with serum produced by The Rockefeller Institute and the Research Laboratory of the Department of Health of the City of New York.

The problem of selecting representative strains was obviously difficult on account of the obstacles encountered in the classification of the meningococci which have been obtained from cases of cerebro-spinal meningitis, from carriers, and from healthy persons. The four representative or standard strains selected included one regular strain, one parameningococcus, and two intermediate strains. One horse was immunized with these four cultures; the other horse was immunized with two cultures of each of the three main groups. These strains represented the groups recognized in the classifications of Dopter,[1] Gordon,[2] Flexner,[3] and others. When only four or six representative strains were used the horse received at each dose an equal quantity of each strain. The dose was increased and adjusted to give rise to approximately the same degree of reaction that was obtained in the immunization with a large number of strains.

Method.

The method of preparing the cultures and immunizing the horses with suspensions of them was similar whether 4, 6, or 60 strains were used for immunization. The cultures were grown on serum dextrose agar slants for 17 to 19 hours at 37°C. and were suspended in 0.85 per cent salt solution. The inoculations were made intravenously

[1] Dopter, C., Étude de quelques germes isolés du rhino-pharynx, voisins du méningocoque (paraméningocoques), *Compt. rend. Soc. biol.*, 1909, lxvii, 74.

[2] Gordon, M. H., Bacteriological studies in the pathology and preventive control of cerebro-spinal fever among the forces during 1915 and 1916. II.—The definition of the meningococcus, *Med. Research Com., Nat. Health Insurance, Special Rep. Series, No. 3*, 1917, 10.

[3] Flexner, S., Mode of infection, means of prevention and specific treatment of epidemic meningitis, *J. Am. Med. Assn.*, 1917, lxix, 639.

without delay. At the beginning of immunization the first dose was a fraction of a single culture. When a large number of strains was used new strains were added as fast as possible, until twenty were injected daily. Two inoculations were made on each day, 1 hour apart, with ten cultures for each inoculation. This was done to decrease the severity of the reaction. The doses were given on 3 successive days followed by a week of rest, after which another series was begun. The cultures and the method of injection were chosen so that the whole series of stock cultures was injected as often as possible. The increase of dosage was dependent upon the temperature reaction after inoculation, the aim being to secure a rise in temperature to 104° or 105°F. 5 hours after inoculation, followed by a fall to normal the next morning.

Trial or complete bleedings were made on the 6th day after every other series of injections. The immunization of the two horses with the four and six representative strains was begun in May, 1918, and was continued for a period of more than 1 year. The largest dose was a total of 60 cc. of suspension, representing the growth from ten slants of culture. The horses were allowed to rest for 2 months, during August and September, 1918. The potency of the serum was tested according to the standard methods described in a previous paper.[4]

Cultures were grown at 37°C. for 18 hours on serum dextrose agar and then suspended in 0.85 per cent salt solution and diluted to a density equal to that of barium sulfate Standard No. 3, or approximately 2,000 million cocci per cc. In all the tests saline solution, normal horse serum, and type serum controls were used, as well as the standard antimeningococcus serum adopted by this laboratory. Although the standard serum has been used during a period of 2 years, it has shown practically no deterioration in agglutination titer.

According to these standard methods the agglutination titer was determined by the maximum dilution in which definite but not necessarily complete agglutination occurred. Reading the limit of definite

[4] Wadsworth, A. B., Kirkbride, M. B., and Gilbert, R., The standardization of antimeningococcus and antipneumococcus serums, *Arch. Int. Med.*, 1919, xxiii, 269.

agglutination, rather than of complete agglutination, has been found to be more accurate and practical. Complete agglutination occurs in a much lower dilution, and if a sufficient number of dilutions of serum are used to grade the degree of reaction accurately, different workers may experience difficulty in reading the complete agglutination. Furthermore, the ability of certain cultures to be agglutinated completely, and that of certain serums to agglutinate, vary. Finally, the percentage of discrepancy between the readings of different workers when the upper limits of agglutination are read and high dilutions are made of the serum is not great, especially if the dilutions which are doubtful are always considered negative, and only the highest dilution which shows definite agglutination is read.

RESULTS.

In Tables I and II are recorded agglutination tests on the serums of the two horses during immunization with four and six strains respectively in comparison with the serum of a horse immunized with 60 strains during a period of 18 months (Table III). The tests were made with the six cultures used in the immunization of the horse mentioned above. The rise in agglutination titer is very rapid during the first few weeks of immunization. Then it is slower until a constantly high titer has been reached, which, however, varies at times quite markedly, doubtless on account of a dose that was too large or too small. The potency as indicated by the agglutination titer with homologous strains was increased three- to tenfold by limiting the number of strains used in immunization.

Agglutination tests have been made with the serums of horses immunized with 4, 6, and 60 strains of meningococci against 77 homologous and heterologous cultures, including also some strains recently isolated from cases, and others reputed to belong, because of their serological reactions, to the fourth group of Gordon, typical strains of which are not commonly found in this country. From Tables IV and V it is evident that many of the heterologous type cultures were agglutinated to an even higher degree than the homologous cultures. Practically all the twelve unclassified cultures which were used in the immunization of the 60 strain horse, and also the twelve additional cultures, none of which was used in the immuni-

TABLE I.

Agglutination Titer of the Serum of Horse 49 during Immunization with Four Strains of Meningococci.

Time of test.	Date.	Agglutination titer tested with different cultures.					
		No. 1B*	No. 10B	No. W30B	No. 46B	No. W60B	No. 79B*
	1918						
Normal serum	May 3	1:50	1:100	1:100	1:50	1:50	1:100
After 2nd series of injections	" 22	1:<200	1:1,000	1:2,000	1:5,000	1:4,000	1:5,000
" 4th " " "	June 10	1:1,000	1:2,000	1:3,000	1:8,000	1:5,000	1:7,000
" 6th " " "	" 26	1:2,000	1:4,000	1:3,000	1:12,000	1:6,000	1:8,000
" 8th " " "	July 15	1:1,000	1:3,000	1:4,000	1:9,000	1:6,000	1:7,000
" 10th " " "	Aug. 2	1:3,000	1:5,000	1:5,000	1:12,000	1:8,000	1:9,000
" 2 mos. rest	Oct. 14	1:1,000	1:1,000	1:2,000	1:10,000	1:2,000	1:4,000
" 12th series of injections	Nov. 1	1:<2,000	1:4,000	1:4,000	1:6,000	1:6,000	1:6,000
" 14th " " "	" 21	1:2,000	1:4,000	1:4,000	1:8,000	1:8,000	1:10,000
" 16th " " "	Dec. 11	1:2,000	1:3,000	1:4,000	1:8,000	1:3,000	1:7,000
" 18th " " "	" 30	1:2,000	1:2,000	1:4,000	1:10,000	1:8,000	1:10,000
	1919						
" 19th " " "	Jan. 8	1:2,500	1:4,000	1:5,500	1:11,000	1:5,500	1:8,500
" 21st " " "	Feb. 1	1:1,000	1:4,000	1:4,000	1:12,000	1:4,000	1:8,000
" 23rd " " "	" 21	1:1,000	1:4,000	1:4,000	1:12,000	1:4,000	1:4,000
" 24th " " "	Mar. 3	1:1,000	1:2,000	1:4,000	1:12,000	1:5,000	1:9,000
" 26th " " "	" 28	1:1,500	1:2,500	1:3,500	1:11,500	1:6,000	1:8,000
" 28th " " "	Apr. 21	1:3,000	1:3,000	1:4,000	1:12,000	1:5,000	1:8,000
" 30th " " "	June 14	1:4,000	1:4,000	1:6,000	1:12,000	1:4,000	1:8,000
" 34th " " "	" 30	1:2,000	1:3,000	1:4,500	1:12,000	1:6,500	1:6,500

* These cultures were not used in the immunization of Horse 49.

In Tables I, II, and III the figures of dilution represent the highest dilution in which agglutination visible to the naked eye took place. < a certain dilution indicates that a lower dilution was not tested. Due to the fact that the end-points of agglutination were not determined in early tests, the tests with serums from May 3, 1918, through Aug. 2, 1918, have been repeated after 1 to 1½ years. The other tests represent the titers obtained within about 3 months after bleeding. Immunization was discontinued from Aug. 2, 1918, through Oct. 14, 1918. When two or more tests were made on the same serum, the average was recorded to the nearest 500, except in Table III.

TABLE II.

Agglutination Titer of the Serum of Horse 40 during Immunization with Six Strains of Meningococci.

Time of test.	Date.	Agglutination titer tested with different cultures.					
		No. 1B	No. 10B	No. W30B	No. 46B	No. W60B	No. 79B
	1918						
Normal serum	May 3	1:<50	1:<50	1:50	1:100	1:50	1:100
After 2nd series of injections	" 22	1:500	1:3,000	1:2,000	1:1,000	1:3,000	1:3,000
" 4th " " "	June 10	1:2,000	1:4,000	1:4,000	1:8,000	1:6,000	1:8,000
" 6th " " "	" 26	1:3,000	1:8,000	1:6,000	1:12,000	1:9,000	1:12,000
" 8th " " "	July 15	1:4,000	1:5,000	1:4,000	1:12,000	1:10,000	1:10,000
" 10th " " "	Aug. 2	1:3,000	1:6,000	1:5,000	1:12,000	1:10,000	1:11,000
" 2 mos. rest	Oct. 14	1:<1,000	1:1,000	1:1,000	1:8,000	1:8,000	1:4,000
" 12th series of injections	Nov. 1	1:2,000	1:1,500	1:3,000	1:8,000	1:8,000	1:10,000
" 14th " " "	" 21	1:2,000	1:1,000	1:4,000	1:10,000	1:4,000	1:10,000
" 16th " " "	Dec. 11	1:2,000	1:4,000	1:2,000	1:10,000	1:5,000	1:8,000
" 18th " " "	" 30	1:2,000	1:2,000	1:4,000	1:12,000	1:4,000	1:10,000
	1919						
" 19th " " "	Jan. 8	1:2,000	1:3,000	1:4,500	1:11,500	1:7,500	1:9,000
" 21st " " "	Feb. 1	1:2,000	1:4,000	1:2,000	1:12,000	1:4,000	1:8,000
" 23rd " " "	" 21	1:2,000	1:4,000	1:4,000	1:12,000	1:8,000	1:8,000
" 24th " " "	Mar. 3	1:2,000	1:2,000	1:5,000	1:12,000	1:9,000	1:8,000
" 26th " " "	" 28	1:2,000	1:4,000	1:8,000	1:12,000	1:12,000	1:8,000
" 28th " " "	Apr. 21	1:3,000	1:4,000	1:7,000	1:12,000	1:7,000	1:12,000
" 30th " " "	May 14	1:4,000	1:6,000	1:6,000	1:12,000	1:6,000	1:12,000
" 34th " " "	June 30	1:2,000	1:2,500	1:4,500	1:12,000	1:6,500	1:6,500

zation of any of the horses, were agglutinated in the serum of Horses 40 and 49 in higher dilution than in that of the 60 strain horse. The most radical difference in the action of the serums of Horses 40 and 49 is with Culture 286, which agglutinated in 1:600 dilution with the serum of Horse 50 (60 strains), in 1:2,000 with the serum of Horse 40, and in 1:200 with the serum of Horse 49.

The serums obtained from horses immunized with four and with six representative strains as compared with the serum of a horse immunized with 60 stock meningococcus cultures thus possessed a higher agglutination titer. This was marked when the serum was tested against the homologous strains; but a high titer was also found when the serums were tested against all the heterologous strains. Evidently, by the use of too large a number of strains the potency of the serum, as judged from the agglutination titer, is sacrificed.

TABLE III.

Agglutination Titer of the Serum of Horse 50 Immunized with 60 Strains of Meningococci for 18 Months.

Date.	Agglutination titer tested with different cultures.					
	No. 1B	No. 10B	No. W30B	No. 46B	No. W60B	No. 79B
1917						
Nov. 14.....	1:700	1:800	1:1,000	1:1,100	1:900	1:1,100

The titer of a polyvalent serum obtained by immunization with 60 strains for 18 months approximated dilutions of 1:700, 1:800, 1:1,000, 1:1,100, 1:900, and 1:1,100 respectively with six representative strains of the different types of meningococci, whereas the horse immunized with these six strains possessed an agglutination titer of 1:4,000, 1:6,000, 1:6,000, 1:12,000, 1:6,000, and 1:12,000 respectively. The horse immunized with only four of the strains possessed an agglutination titer of 1:4,000, 1:4,000, 1:6,000, 1:12,000, 1:4,000, and 1:8,000 respectively, the first and last reactions being heterologous and the others homologous; that is, tests with the cultures used in immunization of the horse. There is thus marked variation in the titer obtained with the different strains, largely owing to the antigenic properties or agglutinability of the cultures. The titer apparently fluctuated to some extent while the horses were in a high

TABLE IV.

Results of Agglutination Tests of Meningococcus Cultures with Experimental Polyvalent Antimeningococcus Serums.

Culture No.	Classification of cultures.		Complete agglutination.*					Maximum agglutination.†				
			Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.		Serum of Horse 49 immunized with 4 strains of meningococci.		Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.		Serum of Horse 49 immunized with 4 strains of meningococci.	
	Gordon nomenclature.	Ordinary nomenclature.	Nov. 14, 1917.	July 15, 1918.	Dec. 30, 1918.	Aug. 2, 1918.	Dec. 30, 1918.	Nov. 14, 1917.	July 15, 1918.	Dec. 30, 1918.	Aug. 2, 1918.	Dec. 30, 1918.
1	Type II	Para.	—‡	1:1,000	1:1,000	1:1,000	1:1,000	—	1:2,000	1:4,000	1:4,000	1:2,000
2	" III	Irregular.	—	1:1,000	1:1,000	1:1,000	1:1,000	1:500	1:4,000	1:4,000	1:8,000	1:8,000
3	" III	"	—	1:1,000	1:500	1:1,000	1:500	—	1:4,000	1:2,000	1:4,000	1:2,000
4B	" II	Para.	—	1:1,000	1:1,000	1:1,000	1:1,000	—	1:8,000	1:8,000	1:8,000	1:4,000
5	Unclassified.	Unclassified.	—	—	—	—	—	—	—	—	—	—
6	Type II	Para.	—	1:1,000	1:500	1:1,000	1:500	—	1:4,000	1:4,000	1:4,000	1:4,000
7	" III	Irregular.	—	1:1,000	1:500	1:1,000	1:500	1:1,000	1:2,000	1:2,000	1:2,000	1:2,000
8	" II	Para.	—	—	—	—	—	1:1,000	1:4,000	1:4,000	1:2,000	1:4,000
9	Unclassified.	Unclassified.	—	—	—	—	—	—	—	—	1:500	—
10	Type III	Irregular.	—	—	1:500	1:500	1:500	1:1,000	1:4,000	1:4,000	1:8,000	1:8,000
30	" III	"	—	1:1,000	1:1,000	1:2,000	1:2,000	1:1,000	1:8,000	1:12,000	1:8,000	1:8,000
31	" III	"	—	1:500	1:500	1:1,000	1:2,000	1:500	1:2,000	1:8,000	1:8,000	1:12,000
32	Unclassified.	Unclassified.	—	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:4,000	1:4,000	1:4,000	1:4,000
33	Type II	Para.	—	1:500	1:500	1:500	—	1:1,000	1:1,000	1:1,000	1:1,000	1:500
35A	" II	"	—	1:500	1:500	1:500	—	1:1,000	1:1,000	1:1,000	1:1,000	1:500
36	" III	Irregular.	—	1:8,000	1:8,000	1:8,000	1:8,000	1:2,000	1:12,000	1:12,000	1:12,000	1:12,000
37	" III	"	—	1:1,000	1:1,000	1:1,000	1:1,000	1:2,000	1:8,000	1:4,000	1:4,000	1:2,000
38A	Types I and III	" Normal.		1:1,000	1:1,000	1:500	—		1:8,000	1:8,000	1:8,000	1:4,000
39	Type I	"	1:500	1:4,000	1:4,000	1:2,000	1:2,000	1:1,000	1:12,000	1:12,000	1:12,000	1:12,000

40	Type II	Para.	—	—	—	—	—	1:500	1:4,000	—	—	1:1,000
42	" III	Irregular.	—	1:1,000	1:2,000	1:1,000	1:1,000	1:2,000	1:8,000	1:8,000	1:4,000	1:4,000
43	" II	Para.	—	—	—	1:500	—	1:500	1:2,000	1:500	1:4,000	1:2,000
44	" II	"	—	1:500	1:500	—	—	—	1:1,000	1:1,000	1:500	1:1,000
45	" III	Irregular.	—	1:500	1:500	1:500	1:500	1:1,000	1:1,000	1:4,000	1:8,000	1:4,000
46	" II	Para.	—	1:4,000	1:4,000	1:4,000	1:8,000	1:1,000	1:12,000	1:12,000	1:8,000+	1:12,000
48	" III	Irregular.		1:500	1:1,000	1:1,000	1:500		1:4,000	1:2,000	1:8,000	1:4,000
49	" III	"	—	—	—	—	—	1:2,000	1:500	1:1,000	1:2,000	1:2,000
50	" III	"		1:2,000	1:2,000	1:4,000	1:1,000		1:12,000	1:12,000	1:12,000	1:12,000
60	" I	Normal.	—	1:4,000	1:2,000	1:1,000	1:8,000	1:500	1:12,000	1:4,000	1:4,000	1:12,000
61	Unclassified.	Unclassified.	—	—	—	—	—	—	1:1,000	1:500	1:1,000	1:1,000
62	Type II	Para.	—	—	—	1:1,000		—	1:4,000	1:4,000	1:4,000	
63	" I	Normal.	1:500	1:2,000	1:2,000	1:2,000	1:1,000	1:1,000	1:12,000	1:4,000	1:8,000	1:8,000
64	" III	Irregular.	—	1:500	1:500	1:1,000	1:500	1:1,000	1:2,000	1:1,000	1:4,000	1:4,000
65	Unclassified.	Unclassified.		—	—	—	—		1:1,000	1:1,000	1:1,000	1:500
66	Type III	Irregular.	—	1:500	1:500	1:2,000	1:1,000	1:2,000	1:12,000	1:8,000	1:12,000	1:12,000
67	" III	"	—	—	—	—	—	—	1:500	1:500	1:500	1:500
68B	" III	"	—	—	—	—	—	—	1:500	—	—	—
69	" III	"	—	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:2,000	1:2,000	1:4,000	1:2,000
70	Unclassified.	Unclassified.	—	1:1,000	1:1,000	1:1,000	1:500	1:500	1:4,000	1:4,000	1:2,000	1:2,000
71	Type III	Irregular.	—	1:500	1:500	1:500	—	1:2,000	1:2,000	1:4,000	1:8,000	1:2,000
72	" II	Para.	—	1:500	1:1,000	1:500	—	1:500	1:2,000	1:2,000	1:1,000	1:500
73	Unclassified.	Unclassified.	—	—	—	—	—	1:500	1:1,000	1:500	1:1,000	1:1,000
74	Type III	Irregular.	—	1:500	1:1,000	1:500	—	1:500	1:2,000	1:2,000	1:8,000	1:2,000
75	" III	"	—	1:1,000	1:1,000	1:1,000	1:1,000	1:2,000	1:8,000	1:12,000	1:8,000	1:8,000
76	" I	Normal.	—	1:1,000	1:1,000	1:1,000	1:500	1:500	1:12,000	1:4,000	1:4,000	1:2,000
77	" I	"	—	1:1,000	1:2,000	1:500	1:1,000	1:500	1:4,000	1:4,000	1:2,000	1:4,000
78	" III	Irregular.	—	1:1,000	1:1,000	1:1,000	1:1,000	1:1,000	1:4,000	1:8,000	1:2,000	1:12,000
79	" I	Normal.	—	1:1,000	1:2,000	1:1,000	1:1,000	1:1,000	1:12,000	1:8,000	1:8,000	1:4,000
80	" III	Irregular.	—	—	1:500	1:500	1:500	—	1:4,000	1:1,000	1:8,000	1:1,000

* The dilution in which the supernatant fluid is clear.

† The standard maximum agglutination titer.

‡ Blank indicates that the culture was not tested; —, that the reaction was not obtained in the lowest dilution tested, 1:500, except as otherwise indicated; namely, 1:200 with the New York Hospital culture and No. 55, and with the cultures in Table V.

TABLE IV—*Concluded.*

Culture No.	Classification of cultures.		Complete agglutination.*					Maximum agglutination.†				
			Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.		Serum of Horse 49 immunized with 4 strains of meningococci.		Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.		Serum of Horse 49 immunized with 4 strains of meningococci.	
	Gordon nomenclature.	Ordinary nomenclature.	Nov. 14, 1917.	July 15, 1918.	Dec. 30, 1918.	Aug. 2, 1918.	Dec. 30, 1918.	Nov. 14, 1917.	July 15, 1918.	Dec. 30, 1918.	Aug. 2, 1918.	Dec. 30, 1918.
81	Unclassified.	Unclassified.	—	—	—	—	—	1:500	1:1,000	1:2,000	1:1,000	1:1,000
82	"	"	—	—	1:500	1:1,000	—	1:500	1:4,000	1:2,000	1:4,000	1:500
84	Type II	Para.	—	—	—	—	—	—	1:1,000	1:500	1:1,000	1:2,000
1B	" II	"	—	1:500	—	1:500	1:500	1:500	1:2,000	1:2,000	1:2,000	1:2,000
10B	" III	Irregular.	—	—	1:500	—	—	1:500	1:1,000	1:2,000	1:2,000	1:2,000
W30B	" III	"	—	1:1,000	1:1,000	1:1,000	1:2,000	1:2,000	1:2,000+	1:8,000	1:8,000	1:8,000
46B	" II	Para.	—	1:2,000	1:4,000	1:2,000	1:4,000	1:2,000	1:12,000	1:12,000	1:12,000	1:12,000
W60B	" I	Normal.	—	1:2,000	1:2,000	1:2,000	1:1,000	1:500	1:8,000	1:8,000	1:8,000	1:4,000
79B	" I	"	—	1:2,000	1:2,000	1:2,000	1:1,000	1:2,000	1:8,000	1:12,000	1:12,000	1:8,000
Type II (Gordon).	" II	Para.		1:2,000	1:1,000	1:1,000	1:500		1:12,000	1:12,000	1:12,000	1:12,000
Type IV (Gordon).	" IV	?	—	1:500	1:1,000	1:1,000	1:500	—	1:2,000	1:2,000	1:2,000	1:2,000
				Mar. 28, 1919.		Mar. 28, 1919.			Mar. 28, 1919.		Mar. 28, 1919.	
105	Type II	Para.	—	—		—		—	1:1,000		1:1,000	
				June 30, 1919.		June 30, 1919.			June 30, 1919.		June 30. 1919.	
New York Hospital.	Type II	Para.	—	—		—		1:200	1:3,000		1:3,000	
55	" II	"	—	1:2,000		—		1:200	1:5,000		1:2,000	

TABLE V.

*Results Obtained with Cultures Received from Dr. McCoy.**

Culture No.	Classification of cultures.			Complete agglutination.†			Maximum agglutination.‡		
				Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.	Serum of Horse 49 immunized with 4 strains of meningococci.	Serum of Horse 50 immunized with 60 strains of meningococci.	Serum of Horse 40 immunized with 6 strains of meningococci.	Serum of Horse 49 immunized with 4 strains of meningococci.
	Gordon nomenclature.	Ordinary nomenclature.	McCoy's classification.	Nov. 14, 1917.	Mar. 28, 1919.	Mar. 28, 1919.	Nov. 14, 1917.	Mar. 28, 1919.	Mar. 28, 1919.
11	Type I	Normal.	Type I	—	1:500	1:200	1:800	1:5,000	1:5,000
123	" I	"	" I	—	1:4,000	1:2,000	1:1,200	1:9,000	1:7,000
128	" III	Irregular.	Not given.	—	1:800	1:2,000	1:1,000	1:10,000	1:12,000
57	" III	"	Type III	—	—	1:500	1:1,200	1:3,000	1:3,000
286	Unclassified.		" II	—	—	—	1:600	1:2,000	1:200
56	Type II	Para.	" II	—	—	—	1:800	1:4,000	1:3,000
138	" III	Irregular.	" IV	—	1:1,000	1:2,000	1:1,000	1:6,000	1:9,000
289	Unclassified.		Indefinite.	—	—	—	1:<200	1:200	1:200
301	Type II	Para.	Type II	—	—	—	1:800	1:2,000	1:3,000
98	" III	Irregular.	" I	—	1:800	1:800	1:1,200	1:7,000	1:5,000
60	Unclassified.		" IV	—	—	—	1:200	1:800	1:800
307	Type III	Irregular.	Not given.	1:200	1:1,000	1:800	1:1,200	1:7,000	1:12,000
136	" II	Para.	Type II	—	1:800	1:800	1:1,200	1:7,000	1:12,000
					Nov. 8, 1919.	Oct. 31, 1919.		Nov. 8, 1919.	Oct. 31, 1919.
55	Type II	Para.	Type II	—	1:800	—	1:800	1:5,000	1:4,000

* These cultures were obtained from Dr. G. W. McCoy, of the Hygienic Laboratory, U. S. P. H.

† The dilution in which the supernatant fluid is clear.

‡ The standard maximum agglutination titer.

state of immunization, but the minimum titer of the serum from the two horses during this stage greatly exceeded the titer of the polyvalent serum from the horse immunized with 60 strains.

Since with a small number of strains, such as would meet the minimum requirements of the standards of potency for the manufacture of antimeningococcus serum in New York State, the agglutination titer may be greatly increased, as compared with previous methods in which a large number of strains was used, the minimum standards of potency should be correspondingly increased. Furthermore, the minimum standards of potency, as determined by the agglutination titer, must be adjusted for each standard culture. Until we have had more experience with the variations in titer of serums from a larger number of horses, it is, of course, difficult to determine practically how much to adjust the standard above or below the minimum agglutination titer obtained during the height of immunization in this investigation.

CONCLUSIONS.

The potency of a polyvalent antimeningococcus serum, as tested by its agglutination titer, was sacrificed by immunization with a large number of strains of the meningococcus. By immunization with a limited number of representative strains, four or six, carefully selected on account of their antigenic and agglutination properties, the potency was increased three- to tenfold without sacrificing the polyvalency; that is, as tested with at least 70 heterologous strains of the meningococcus.

The agglutination titer, unfortunately, is not an entirely satisfactory criterion of therapeutic potency, but it is the only practical method available that determines also the polyvalent action of antimeningococcus serum.

PURIFICATION AND CONCENTRATION OF ANTIGENS FOR COMPLEMENT FIXATION BY METHODS OF DIALYSIS, ADSORPTION, AND EXTRACTION.*†

By AUGUSTUS B. WADSWORTH, M.D., and FRANK MALTANER.

(*From the Division of Laboratories and Research of the New York State Department of Health, Albany.*)

(Received for publication, July 12, 1920.)

The complement fixation test of Bordet and Gengou with bacterial antigens on the serum of highly immunized animals has been of value in the identification of bacterial species, but it has never been a practical aid in the diagnosis or prognosis of bacterial infection in man. Although the antigens which have been prepared from syphilitic tissue and even from certain normal tissues have proved to be of great value in the diagnosis of syphilis, none of the antigens which have been prepared from pure cultures of the spirochete isolated from syphilitic tissue has been reliable. On the contrary, they apparently lack reactive capacities that are characteristic for this disease. Thus the nature of the complement fixation reaction of Wassermann is obscure. The so called anticomplementary or nonspecific properties of antigens which have been prepared from pure cultures of the incitants of the bacterial infection have made it difficult or impossible to standardize the test so that the characteristic reactions with the blood serum from cases of infection could be detected or studied to determine their diagnostic or prognostic value.

Prior to his work on syphilis Wassermann studied the complement fixation reaction in tuberculosis, but failed to prepare satisfactory antigens from tubercle bacilli. Recently a number of investigators have reported diagnostic reactions in so many cases of tuberculosis that there can be no question that it is possible to prepare antigens

* Presented at the 35th annual meeting of the Association of American Physicians, Atlantic City, N. J., May 4, 1920.

† An abstract of this paper will appear in the *Proc. Soc. Exp. Biol. and Med.*

from the tubercle bacillus, and in certain stages of the disease the blood serum gives positive reactions with them; but it has not been possible to standardize the test so that it is reliable in the diagnosis of the disease or in the study of the development of immune reactions in different stages of the disease.

Work was begun in 1917, with the view of so standardizing the preparation of the antigens that it would be possible, first, to study the development of immune reactions in experimental tuberculosis in animals and, later, to determine the diagnostic value of the test in human cases of the disease. New methods of dialysis and adsorption were used in the preparation of the antigens by which the antigenic substances were greatly purified and concentrated. The purpose of this preliminary paper is to record the new methods.

Preparation of Standard Immune Serums.

All the cultures were made in a beef infusion broth containing 1 per cent of Fairchild's peptone, 0.5 per cent salt, 0.5 per cent glycerol, and titrated to react 0.5 per cent acid to phenolphthalein. The medium was sterilized fractionally in the Arnold sterilizer. Mass cultures were grown in quart or pint Blake bottles at a temperature of 37.5°C. With some cultures the growth was taken for study as soon as the pellicle had become fully developed, and with others as soon as growth had ceased. After filtration the residue of tubercle bacilli was washed with saline solution and dried partially by suction on the filter. It was then desiccated over concentrated sulfuric acid. This material was then extracted with different substances, such as carbon disulfide, ether, chloroform, and carbon tetrachloride. The fat-free residues from these extractions and also the untreated bacilli were extracted with distilled water, salt solution, sodium hydroxide, hydrochloric acid, and glycerol. The various extracts, together with the filtrates from the cultures, were tested as antigens. This technique was applied to three different strains of tubercle bacilli, a human virulent strain, a human avirulent strain, and a strain of bovine origin. In the production of immune serums by animal inoculation tuberculous organs became available which were extracted by various means and tested for antigenic properties.

The preparation of standard immune serums with these three cultures was a prolonged investigation of experimental infection and immunization of a large number of animals.[1] It involved not only the testing of the inoculated animals at frequent intervals but also a study of the precipitation of the globulin fraction of serum in an attempt to concentrate the antibodies. Finally, sufficiently active immune serums were obtained from horses with all three strains of the tubercle bacillus to give complete fixation of complement with the antigens which were arbitrarily selected to start the work. A single bleeding of any of the horses gave a sufficiently large quantity of serum for the titration of the comparative value of all the antigens.

Antigenic Properties of Different Fractions of the Culture and Methods of Purification and Concentration.

The results of the experiments in which the different fractions of the culture were tested with the standard immune serums must also be briefly summarized. The preparation of antigens by extraction of tuberculous tissues was unsatisfactory. The culture filtrates were so anticomplementary that they could rarely be used. Of all the extracts of the tubercle bacillus which have as yet been tested in our experiments, those with glycerol and, especially, distilled water produced the most active antigens. These active antigens were selected for further study by methods of dialysis and adsorption.

Animal charcoal and globulin (horse serum) were used to adsorb the substances possessing antigenic action. When animal charcoal was added the preparations lost their antigenic activity, but it is impossible to say whether this activity was adsorbed on the charcoal or destroyed as it has been as yet impossible to recover from the animal charcoal any of the antigenic properties. When globulin was added and precipitated with carbon dioxide and the precipitate extracted with alcohol, the substances possessing antigenic action with tuberculous immune serum were adsorbed and recovered in purified and concentrated condition. The so called anticomplementary action which all the original antigens possessed could not be

[1] This work was successfully carried on by Miss B. Johnston and will be recorded more fully in another paper.

detected in the lowest dilutions of the alcoholic extract (1:10) which could be used. The lytic action which some of the original antigens possessed also disappeared.

The technique that was used may be briefly summarized as follows: The original antigen was dialyzed, and horse serum, one part to twenty parts of antigen, was added and the mixture allowed to stand in the incubator for ½ hour. The globulin was then precipitated by passing purified carbon dioxide gas free from hydrochloric acid through the mixture for ½ hour at 37°C. The globulin precipitate was collected, and by shaking it with alcohol the antigenic substances were extracted. The alcoholic extract was concentrated in a vacuum. All the titrations were made in quantities of one-tenth of the original Wassermann test.

In order to eliminate all the dialyzable substances which prevent the adsorption of the antigenic material by globulin, the culture filtrates were dialyzed for at least 5 days. Tests of the antigenic properties at different stages of the process revealed the fact that after dialysis the filtrate became active as an antigen, and lost its anticomplementary action. After precipitation with carbon dioxide and extraction of the globulin precipitate much more active antigens were obtained. The most active antigens, however, were those obtained by this method from the distilled water extracts of the tubercle bacillus.

The following illustrates the degree of concentration and purification. Broth filtrates which were so anticomplementary that they gave no specific fixation, after dialysis were not anticomplementary and 0.02 cc. gave one unit of antigen; that is, complete fixation with the standard quantity of immune serum—two units as determined by a previous titration with one of the crude antigens which were used to standardize the immune serums. After adsorption and concentration 0.0003 cc. gave a unit of antigen. The aqueous extract antigens were anticomplementary in doses of approximately 0.05 to 0.01 cc. The results of purification and concentration of these antigens are even more striking. Before dialysis 0.003 cc. of one of them contained one unit of antigen; after dialysis it was no longer anticomplementary but owing to the increase in volume during dialysis 0.005 cc. contained one unit of antigen. After concentration 0.00015 cc. contained one unit. Expressed in terms of antigenic units per cc. this is an increase from 333 to 6,666.

The results of preliminary experiments with antigens which are used in the diagnosis of syphilis indicate that their antigenic properties may also similarly be purified and concentrated. These new methods thus open up possibilities for more precise study of many phases of infection and immunity than has hitherto been possible.

SUMMARY.

Antigens were prepared from the culture filtrates of tubercle bacilli and by extraction of washed and dried organisms with organic and aqueous solvents and from tissues of organs showing tuberculous lesions.

A comparison of these preparations by means of the complement fixation test showed that the aqueous extracts were most active antigenically.

The antigenic activity of the aqueous preparations and also of the slightly active culture filtrates was increased by means of dialysis and adsorption with serum globulin followed by extraction with alcohol and concentration of the alcoholic extract *in vacuo*.